THE ROLE OF THE IMMUNE RESPONSE IN PERIODONTAL DISEASE

BY

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To my parents

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KEY TO ABBREVIATIONS

ADCC	antibody-dependent cell-mediated cytotoxicity
CAB	counts above background
CFU	colony-forming-units
CMI	cell-mediated immunity
Con A	concanavalin A
CPM	counts per minute
CRBC	bovine erythrocytes
^{3}H-TdR	tritiated thymidine
i.p.	intraperitoneally
LE	Lancefield extraction
LPS	lipopolysaccharide
LRI	Laurell rocket immunoelectrophoresis
MIF	macrophage inhibition factor
OAF	osteoclast-activating factor
PHA	phytohemagglutinin
PMN's	polymorphonuclear leukocytes
RIA	radioimmunoassay
RID	radial immunodiffusion
SDS	dodecyl sodium sulfate
TSBS	tryptic soy broth supplemented

Abstract of Dissertation Presented to the Graduate Council of the University of Florida in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy

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The overall aim of this work was to analyze the Balb/c mouse strain for its efficacy as a model for periodontal disease. Once this aim was accomplished, the studies were extended to define the role of the immune response to a known periodontopathogen, Actinomyces viscosus T14V, in orally superinfected animals.

In order for the Balb/c mouse strain to be an adequate animal for periodontitis study with A. viscosus, three requirements had to be met. First, the organism had to be capable of colonizing in the murine oral cavity. This colonization had to be specific for the tooth surface and control animals could not contain the bacteria in their normal flora.

Second, the mouse had to be able to respond immunologically to a bacterial insult with A. viscosus T14V. This response had to be both humoral, as analyzed in the serum, and cell-mediated, as detected by the lymphoblast assay. Third, the mouse strain had to be capable of developing the characteristic bone loss of periodontitis upon superinfection with A. viscosus T14V. The bone loss had to be significantly greater than that seen in uninfected animals with aging. The Balb/c mouse strain met these three requirements and thus was acceptable as a model system for periodontal disease.

When mice were placed on a hard, mouse-chow diet, and superinfected with varying doses of A. viscosus T14VJ1, all mice became infected, though at a low level. A cell-mediated response to the bacteria was detected in only the low-dose inoculated animals and only the low-dose mice developed a large amount of bone loss. Little serum antibody to the bacteria was detected in low-dose mice.

When mice were placed on a soft, high-carbohydrate diet and superinfected with varying doses of A. viscosus T14VJ1, all mice became infected at a level a hundred fold higher than the animals on a hard diet. A cell-mediated response to the bacteria was detected only in the low dose inoculated animals and only the low-dose mice developed bone loss. No serum antibody to the bacteria was detected in any animal.

The same response to the bacteria was detected in the cervical lymph nodes, which are the draining lymph nodes for the gingival tissue. Cell separation techniques revealed that the B cell was the cell type responding to the bacterial antigens.

The results suggested that the development of an immune response to the periodontopathogen, A. viscosus T14V, was essential for bone loss to occur in superinfected mice, A. viscosus T14V infection selectively activated B cells and that this response could be monitored locally and systematically.

INTRODUCTION

The components and products of the microbiota of dental plaque are major factors in the pathogenesis of periodontal disease (periodontitis). Some of these components contribute to the development of periodontal disease by direct action on appropriate substrates in periodontal tissues. On the other hand, as the plaque microbiota and its components and products penetrate the epithelium of the gingival sulcus and even the deeper tissues of the periodontium, they stimulate the immune mechanisms of the host and elicit immunological responses that are involved in the pathogenesis of periodontal disease.

The aim of this study was to develop a model system in which to study the host response to an oral infection of a known periodontopathogen, Actinomyces viscosus T14V. The aim was extended to analyze the specific responding cell types in the host. Finally, the periodontium was analyzed to determine the host response in the localized inflammation in the mouse model system.

Periodontal Disease

Periodontal disease is a general term used to describe a series of chronic inflammatory states of progressively increasing severity localized at the region between the teeth and gums. The disease state is initiated and maintained by the overgrowth of oral bacteria indigenous to the dental plaque present on tooth surfaces at the gingival margin (1, 2, 3, 4). Clinically, the disease is first recognized as gingivitis, a reversible state characterized by swelling and redness of gingival tissue. In the continued long absence of oral hygiene, the disease progresses to its most advanced state, termed periodontitis. This condition is characterized by massive tissue destruction, the formation of periodontal pockets, and alveolar bone resorption leading to a loss in structural support for the teeth (5).

Histologically, the normal junctional epithelium is several cells thick and is in contact with the enamel surface throughout. In between the junctional epithelial cells, a few polymorphonuclear leukocytes and mononuclear cells can be found (6, 7). These cells make up less than 3% of the volume of the epithelium. Immediately below the junctional epithelium is a plexus of blood vessels. A dense accumulation of collagen surrounds the vessels and makes up the bulk of the connective tissue (8, 9). Deeper in the connective tissue, blood, lymphatic vessels and nerves are present as are a few isolated neutrophils, lymphocytes and macrophages (10).

The sequence of changes which occur during the development of periodontal disease in man has been divided into four stages: initial, early, established and advanced lesions (11). The initial stage consists of increasing numbers of neutrophils and a slight loss of collagen (12). The neutrophils increase so that they occupy about 30% of the volume of the junctional epithelium. With time, the blood vessels just below the junctional epithelium become dilated and increasingly permeable (13). Collagen content of the connective tissue decreases by 44% and the volume is occupied by neutrophils, large mononuclear cells and small lymphocytes.

After a week of plaque accumulation the initial lesion evolves into the early lesion. The hallmark is the accumulation of large numbers of lymphoid cells as an infiltrate in the connective tissue. The lymphoid cells now make up nearly 75% of the total number of cells in the inflamed connective tissue (14). The collagen loss increases to 40% and fibroblasts have taken on cytopathic alterations (15). The lymphoid cells in the inflamed areas are about equally divided between small and medium lymphocytes with a very small number of blast cells. There are few lymphocytes with a very small number of blast cells. Few lymphocytes exhibit surface immunoglobulin (16).

The next phase is termed the established lesion and develops about 3 weeks after the beginning of plaque accumulation (11). The primary feature of this lesion is the vast

amount of plasma cells in the inflamed connective tissue (11, 17). The plasma cells appear not only in the area that was inflamed in the early lesion but also in clusters around the vessels and deep within the connective tissue. Plasma cells range from immature to mature to degenerating. Immunoglobulins are clearly present in the plasma cells (16, 18). Complement components are also present. Lymphocytes are found near fibroblasts that are clearly abnormal. Neutrophils are especially prevalent in the epithelium and can be found in the connective tissue (19). Free neutrophil lysosomal granules can also be found. Blood vessels are now particularly prominent with the vessels widening and changing course during gingivitis. Collagen destruction is wide spread. Pathologic alterations are apparent at the junctional epithelium. A pocket epithelium is now formed with the loss of numbers of epithelial cells and accumulation of inflammatory cells (20). Bacteria are not seen between epithelial cells or in the connective tissue (19).

The established lesion may last for months to years and then may progress into the advanced lesion. The distinguishing feature of this state is the breakdown of the junctional epithelium, connective tissue and bone loss (11). An acute vasculitis is present with an influx of numerous plasma cells, lymphocytes and macrophages. The plasma cells have 78% IgG, as surface immunoglobulins (16). The collagen destruction in the inflamed area is nearly complete. Osteoclastic bone resorption occurs especially along vessels

entering the alveolar crest. A preponderance of plasma cells with neutrophils is present.

Cells in Periodontal Disease

In human periodontal disease, neutrophils (PMN's) can play a protective and destructive role. When these cells are depressed in number, periodontal disease is more severe (21, 22, 23). Periodontosis (juvenile periodontitis) and severe periodontitis patients have altered neutrophil functions. These findings offer presumptive evidence that PMN's are normally protective. Since neutrophils contain substances capable of degrading tissue components, they may also cause local tissue breakdown when they are plentiful and functional. The evidence for destructive potential is twofold. Electron microscopic observations show that PMN's release lysosomal granules into periodontal tissue. Lesions have been produced by injecting PMN's or their granules into tissue (24, 25). Dental plaque and cellular components of A. viscosus promote the selective release of lysosomal enzymes from human peripheral blood polymorphonuclear leukocytes (26). In man, the presence of antibodies specific for oral bacterial antigens in the sera of individuals with periodontitis and the large number of plasma cells in inflamed gingiva suggest that antibodies may be involved in the maintenance and progression of the disease (27). Most of the plasma cells synthesize immunoglobulins of the IgG class but IgM and IgA producing cells can also be found in

the gingiva. The large amount of IgG produced suggests a second set reaction which is not surprising with the proposed continuous exposure of bacterial antigens to the host.

The specificity of the immunoglobulin present in the inflamed gingiva and that produced by gingival plasma cells locally remains unclear although a portion is specific for plaque-derived microorganisms (28, 27). Sections of inflamed gingiva have the capacity to specifically bind microorganisims, demonstrating the presence of antibody with specificity for the antigens of these organisms. Immunoglobulin obtained frcm inflamed gingiva can form immune complexes with antigens of plaque microorganisms (29). There is also evidence for significant quantities of circulating antibody to oral microorganisms in humans with periodontitis (30, 31).

Individuals exhibiting periodontitis give a positive immediate hypersensitivity reaction when skin tested with an antigenic preparation of A. viscosus (32). Indirect evidence has been found for the presence of antigen-antibody complexes of gingival leukocytes by complement binding. Such evidence suggest antibody specific for oral antigens and a possible method of PMN activation other than bacterial phagocytosis (33).

It appears that cell-mediated immunity (CMI) may play a significant role in the pathogenesis of periodontal disease. In general the responses elicited correlated with the extent of periodontal disease, although certain important

exceptions have been demonstrated. In 1970, Ivanyi and Lehner (34) studied the proliferative response of human peripheral blood lymphocytes to a variety of bacterial antigens found in dental plaque in patients with varying degrees of periodontal disease. Patients were divided into four groups according to the severity of disease: no disease (controls), gingivitis, moderate periodontitis, and severe periodontitis. A correlation was observed between lymphocyte proliferative response to plaque antigens and severity of periodontal disease in all groups with the exception of patients with severe periodontitis. In that group, no significant incorporation of tritiated thymidine (^{3}H-TdR) by allegedly sensitized lymphocytes was seen. In a second study, the same investigators examined lymphocyte stimulation by, and the antigenicity of, sonicates of plaque (35). The authors reported that plaque from clinically healthy patients could stimulate lymphocytes from patients with periodontal disease whereas, autologous plaque or plaque from diseased patients failed to stimulate lymphocytes from patients without disease. These results suggested that the response of lymphocytes in patients with periodontal disease was due to prior sensitization of cells, rather than to a difference in the antigenicity of the plaque.

Studies by Horton et al. (36) confirmed the earlier results. Horton and coworkers measured ^{3}H-TdR incorporation by peripheral lymphocytes in response to plaque and salivary antigens in three groups, the first with minimal gingivitis,

the second with moderate gingivitis to incipient destructive periodontitis, and the third with established destructive disease. Lymphocytes were exposed to both autologous and homologous plaque and whole saliva. The authors found that both plaque and saliva from patients with and without disease contained material which could elicit a proliferative response. In agreement with earlier studies they found that the degree of blastogenesis was related to the severity of disease. They also observed a lack of response of fetal cord lymphocytes following exposure to plaque antigens which suggested that sensitization of lymphocytes was required for the response to be observed. They suggested that the sensitization occurred throughout life.

The finding that serum from patients could modify proliferative lymphocytic responses to plaque antigens has been described by Ivanyi and coworkers (37). They found that when sensitized lymphocytes were cultured in media with serum from patients with varying degrees of periodontal disease, the response of the lymphocytes could be altered. Whereas serum from patients with mild to moderate periodontal disease stimulated proliferation, serum from patients with severe periodontal disease inhibited the reaction. They further reported that the stimulating effect could be decreased if sera were absorbed with stimulating antigen prior to testing, suggesting a role for antibody. In fact, they observed a positive correlation between the level of lymphocyte stimulation and anti-Veilonella antibody titer.

In contrast, absorption of serum with Veilonella has no effect on the inhibition of proliferation using serum from patients with severe disease.

A more recent study by Horton and colleagues (38) suggests that sensitization of lymphocytes to plaque antigens may occur in utero. They found that fetal cord lymphocytes from mothers with significant periodontal disease were sensitized to plaque antigens as measured by proliferative responses.

Thus, studies of lymphocyte proliferative response to plaque antigens indicate that CMI may play a role in periodontal disease. Further, they strongly suggest that serum factors may be important in modifying these responses. It is noteworthy that each of these studies has measured the proliferative response of peripheral blood lymphocytes to plaque antigens.

Some of the most significant series of findings suggesting an immune mechanism for tissue destruction in periodontal disease have been those measuring lymphokine production. The first mediator measured from sensitized lymphocytes upon exposure to plaque antigens was macrophage inhibition factor (MIF) (39). This finding, coupled with the report by Wahl et al. (40) and Gordon (41), suggests that once localized, macrophages can be activated to release collagenase, an enzyme which is potentially destructive to the periodontium. Two other lymphokines may play a significant role in periodontal disease. Horton and coworkers (42) found that when

sensitized lymphocytes were reacted with antigen they released a mediator capable of activating osteoclasts. Using fetal rat bone cultures, they found the supernatant fluid from cultures of human peripheral blood lymphocytes which had been stimulated by phytohemagglutinin (PHA) or plaque antigens contained a substance which caused ^{45}calcium release from labeled bone, as well as an increase in the number of osteoclasts present. The factor has been called osteoclast-activating factor (OAF). Using a simular culture system, Horton et al. (43) found that PHA or plaque-activated lymphocyte cultures contained a substance (lymphotoxin) which decreased the production of protein by fibroblasts. The significance of this lymphotoxin in periodontal disease is that it may be a mechanism for direct destruction of connective tissue elements. Thus, lymphokine experiments support a role for CMI in periodontal disease.

The third group of studies demonstrating a role for CMI in periodontal disease is cytotoxicity studies. Although this function has not been studied as extensively as proliferation or lymphokine production, the results are consistent with these other measurements of CMI. Using a nonspecific cytotoxic assay developed by Perlmann and Holm (44), Ivanyi et al. (37) showed that plaque antigens could elicit a cytotoxic response mediated by sensitized lymphocytes of xenogeneic target cells. Sensitized lymphocytes were stimulated with antigen or PHA, and the mixture allowed to incubate.

Target cells (^{51}Cr-labeled CRBC) were then added and cell death was measured by ^{51}Cr-release. Ivanyi et al. found a correlation between this form of cytotoxic response and the severity of periodontal disease.

Using lymphocytotoxicity to measure specific lymphocyte stimulation, Movius and coworkers (45) described cytolysis of gingival epithelial target cells mediated by allogeneic lymphocytes. Cytolysis was compared between patients with and without periodontal disease using target cell viability as an endpoint. The results indicated that cytolysis of epithelial target cells was more marked using lymphocytes from patients with severe disease than from patients with little or no disease. It should be noted that no antigen was added to the culture of epithelial target cells and lymphocytes. Unfortunately, the authors did not include another form of allogeneic cells as a specificity control. They mention that previous work and other controls done in a different experiment suggest that lysis due to allogeneic differences was unlikely.

It may be speculated that antibody-dependent cell-mediated cytotoxicity (ADCC) plays a role in periodontal disease. ADCC occurs when target cells, pretreated with antibody, are reacted with nonimmune lymphoid effector cells (46). It is believed that the effector cell binds to the Fc portion of the bound antibody and is then activated to mediate lysis (47). ADCC has been described in a variety

of model systems, although never with respect to periodontal disease. Since all of the elements for ADCC--effector cells, antibody, and target antigen--are present in the periodontal environment, studies of ADCC in the diseased process may be of significance.

The findings of Ivanyi *et al.* (37) that serum from patients with periodontal disease could modify CMI in response to plaque antigens is consistent with other findings of the ability of serum to modify CMI. The ability of serum factors to modify CMI has been studied using two of the *in vitro* parameters previously discussed, blastogenesis and cytotoxicity. Using either assay system, it appears that three elements in serum have the capacity to alter CMI: antibody, antigen, and antigen-antibody complexes. In addition, complexes formed in excess of antigen or antibody appear to have opposite effects on CMI in response to a specific antigen.

Actinomyces are especially potent lymphocyte stimulators and are thus implicated as important etiologic agents in the disease process (48). Individuals who allow plaque to accumulate exhibit an increased lymphocyte transformation response to *Actinomyces viscosus*. This response returns to normal upon the return or oral hygiene (49).

Actinomyces viscosus in Periodontal Disease

There can be little doubt that gingivitis and periodontitis are infectious diseases of bacterial origin. The

evidence comes from the findings that A) germ-free animals do not have periodontitis (48, 50, 51), B) animals and man, in whom the oral flora has been mechanically or chemotherapeutically removed or suppressed, show disease remission (52, 53, 54), C) human gingival inflammation occurs only after the accumulation of plaque on the teeth (55, 56, 57), and D) experimental animals infected by certain microorganisms from human periodontal diseases develop periodontal disease (58, 59, 60, 61).

The mechanisms by which plaque bacteria cause periodontal inflammation is unclear, but before a cause and effect relationship can be established between any oral bacterium and periodontal disease, two requirements must be satisfied. First, the bacterium must possess the ability to colonize tooth surfaces at the gingival margin. Second, the bacterium must produce specific microbial products that act directly or indirectly to initiate a sequence of events that leads to clinical periodontal disease. Based on these criteria, the gram positive filamentous bacterium Actinomyces viscosus has been designated a strong candidate as an etiological agent for human periodontal disease (62, 63).

Several reports have demonstrated the association of A. viscosus with dental plaque and the ability of these bacteria to colonize tooth surfaces. Actinomyces viscosus, along with other filamentous organisms normally represent about 1% of the cultivable flora of supragingival dental plaque and about 20-40% of the total volume of plaque (64).

Studies on experimental gingivitis in man have shown that, after a few weeks, Actinomyces became the predominate cultivable organisms in mature and calcifying plaque (64, 65).

When hamsters free of disease are housed with, fed fecal pellets from, or receive plaque transfers from hamsters whose plaque is rich in A. viscosus, they too become infected and manifest signs of periodontal disease. Transmission of disease is blocked by antibiotics and is suppressed by various antimicrobial agents. A. viscosus has been isolated in large numbers from the heavy accumulations of dental plaque that develop in these previously uninfected animals. If an uninfected hamster is infected with a pure culture of A. viscosus, the development of periodontal disease results. Gnotobiotic rats mono-associated with A. viscosus also develop periodontitis (60). Therefore, infection by A. viscosus alone is sufficient to induce periodontitis.

Although initial interest in the role of Actinomyces sp. in periodontitis arose from the study of transmissible periodontitis in experimental animals, further interest in A. viscosus and its relationship to periodontal disease was a result of its repeated isolation from humans with signs of the disease (66, 67, 68, 69). A. viscosus has also been isolated in large numbers from the dental plaque of children with Down's syndrome, a population severely affected by periodontal disease (70).

Our studies have focused upon one particular human isolate of A. viscosus, strain T14V (71, 72). In germ-free rats, strain T14V has been reported to produce extensive plaque, root surface caries and bone destruction characteristic of periodontal disease.

At least two published reports contain data indicating that extracts of A. viscosus contain glycoproteins which selectively stimulate B-lymphocytes in vitro (73, 74). Both hard and soft tissue destruction have been shown to occur in vitro in the absence of immune mechanisms. Culture supernatant fluids from A. viscosus have been shown to activate release of ^{45}Ca from fetal rat bones (75). The active factor appears to be a non-dialyzable, negatively charge molecule with a molecular weight greater than 10,000 daltons. Activation of osteoclasts of A. viscosus culture supernatant fluids apparently does not occur via a direct effect on the osteoclasts but rather through activation of prostaglandin synthesis as $10^{-4}M$ indomethacin inhibited the ^{45}Ca release (76). Taichman and coworkers have reported the selective release of lysosomal enzymes from human peripheral blood PMN's by dental plaque and cellular components of A. viscosus (77, 78, 79). The data suggest that the active principal resides in the bacterial cell wall.

MATERIALS AND METHODS

Bacterial Strains

A. viscosus T14V was obtained from B. F. Hammond, University of Pennsylvania, Philadelphia. Cultures were stored either as lyophilized stocks or as multiple frozen stocks at -30°C in Tryptic Soy Broth (Difco Laboratories, Detroit, MI) containing 20% glycerol.

Media and Growth

Batch cultures were grown in Tryptic Soy Broth without dextrose (Difco) supplemented with 0.1% yeast extract (Difco) and 1% glucose (TSBS). Cultures were incubated under microaerophilic conditions (90% N_2-10% CO_2) at 37°C in a Psycrotherm (New Brunswick Scientific Co., New Brunswick, NJ) shaker incubator (150 rpm).

Infection and Detection

Balb/c female mice (Charles River Labs, Wilmington, Mass.), 6-8 weeks old, 6 per cage, were fed either Purina mouse hard-chow (Ralston Purina Co., St. Louis, MO) or a soft low fat, high carbohydrate diet made up of 40% sucrose, 15% wheat flour, 32% skim-milk powder, 2% vitamin-mineral

protein supplement and 5% Brewer's yeast (E. Smith, Salem, NH). Mice were infected with 1.6 x 10^7 A. viscosus T14VJ1 cells (a Streptomycin resistent variant of T14V) in 50 µl, using a pipet to place the bacteria in the mouth. The animals were then left without water overnight and the protocol repeated two more days. The mice were then fed and watered ad libitum with the appropriate diet. The mice were sacrificed and the three molars of the right mandible and the three molars of the left maxilla of the infected mice were extracted and ground in a tissue homogenizer in Tryptic Soy Broth. The teeth were ground for 30 seconds, diluted and then 0.1 ml was plated in duplicate on Columbia CNA agar (Difco, Detroit, MI) with 200 µg/ml streptomycin sulfate and 150 mg/l sodium fluoride. The plates were incubated at 37°C for 2 days and the colony forming units (CFU) were counted.

Immunization

Mice were immunized with 100 µg of A. viscosus T14VJ1 whole cells in complete Freund's adjuvant intraperitoneally and then boosted in the alternating weeks with the same concentration of antigen with adjuvant. The mice were then sacrificed after the fourth week. Spleens were removed for evaluation of CMI response and the mice were bled for estimation of a humoral response.

Antigen Preparation

To prepare antigens, A. viscosus T14V were grown in Tryptic Soy Broth without dextrose (Difco, Detroit) supplemented with 0.5% glucose and 0.1% yeast extract to late exponential phase of growth. Cells were washed twice with saline and centrifuged at 5,000 x g for 10 minutes. A 10% suspension was prepared and subjected to oscillation at maximum energy level with a Sonic 300 Dismembrator (Artek Systems Corp., Farmingdale, NY) equipped with a large probe for 1 hour. The suspensions were sonicated for 2-minute intervals with cooling periods of 10 minutes. The broken cells were centrifuged at 1,000 x g for 15 minutes to pellet the remaining whole cells. Unbroken whole cells were resuspended in saline and treated as above for additional cell breakage. The supernatant fluids were centrifuged at 48,000 x g for 15 minutes to pellet the crude cell walls. The resulting supernatant fluids were exhaustively dialyzed against water and lyophilized. The crude cell walls were repeatedly washed with water. The resulting wall preparations were washed with 0.1% dodecyl sodium sulfate (SDS) to remove membrane fragments. After further washings with water, the cell preparations were lyophilized. All procedures were performed at 4°C.

Lymphoblast Assay

Spleen or cervical lymph node cell suspensions were prepared by cutting the tissues into small pieces and

forcing them through a tightly-meshed screen with a spatula. The screen was washed with 12 ml RPMI 1640 (Gibco, Grand Island, NY), 4°C. The cells in the RPMI were then drawn up with a 20 gauge needle, and the cells were forced out. They were then rapidly drawn up again and the needle was replaced with a 25-gauge needle, and the cells were forced out into a tube containing 40 ml RPMI, and were centrifuged at 1200 rpm for 8 minutes at 4°C. The washed cells were then resuspended in 50 ml RPMI and recentrifuged. The cells were then resuspended in 20 ml RPMI, supplemented to contain 1% antibotics, 5% human serum (Gibco, Grand Island, NY). Cell counts and viabilities were determined by hemocytometer and trypan blue exclusion. The cells were diluted to 10^6 cells per ml with 95% viability by Trypan blue exclusion.

Blastogenesis was performed by adding 2 x 10^5 cells in 200 µl to wells of microtiter trays (Cooke Engineering Corp., Alexandria, VA) containing quadruplicate antigen or mitogen preparations at three concentrations. The trays were covered and incubated for 72 hours at 37°C in an atmosphere of 5% CO_2-95% air. For the final 18 hours of incubation, 1 µCi of methyl-^{3}H-thymidine (New England Nuclear, Boston, MA) was added to each culture in order to estimate DNA synthesis. Lipopolysaccharide (LPS) of E. coli 055:85 prepared by the Westphal method was purchased from Difco Laboratories, Detroit, MI. Phytohaemagglutinin-P (PHA) and Concanavalin A(ConA) were obtained from the same source.

The cell cultures were processed using an automatic harvesting device (Otto Hiller Co., Madison, WI) and were collected on glass fiber filters (Reeve Angel, Clifton, NJ) and washed with distilled water. After overnight drying at 37°C, the filters were processed for liquid scintillation in 4 ml ACS (Amersham, Arlington Heights, IL) and counted on an Isocap 300 Liquid Scintillator (Searle Analytic, Inc., North Miami, FL).

Counts above background (CAB) were defined by mean counts per minute (CPM) of stimulated wells minus background CPM minus mean CPM of unstimulated saline control cultures.

Cell Separation

T cells were separated on a nylon wool column by the method of Julius et al. (80). Sterile nylon wool in LP-1 Leuko-Pak Leukocyte Filters (Fenwal Laboratories, Morton Grove, IL) was used after soaking in saline for 2 hours with 3 changes and repeating the procedure with distilled water. The nylon wool was dried in a 37°C incubator for 3 days. About 0.6 gram aliquots of nylon wool were packed into the barrels of 10 ml plastic syringes (Menaject, Sherwood Medical Industries, Inc., St. Louis, MO). The filled barrels were placed in aluminum foil and autoclaved. Before using, the columns were washed with 20 ml of saline containing 5% heat-inactivated fetal calf serum and placed at 37°C for 1 hour. This buffer was used throughout the procedure.

Cell suspensions of 2 ml were loaded onto the columns at 5 x 10^7 cells per ml and were washed into the columns with 1 ml of buffer (37°C). The columns were replaced in the syringe covers and placed at 37°C for 45 minutes. The columns were then washed slowly with 37°C media and 25 ml of effluent was collected. The cells were washed and re-suspended into the RPMI buffer. This procedure routinely yielded 35 ± 8% of the starting cells.

Mouse anti-theta ascites fluid (Litton Bionetics, Inc., Kensington, MD) was produced in ARR/Jackson mice immunized with thymocytes from young C3H/HeJ mice. Splenocytes at a total concentration of 2 x 10^7 cells were incubated with the anti-theta ascites fluid (1/8) and guinea pig complement (1/10) (Litton Bionetics, Inc. Kensington, MD) for 1 hour at 37°C in the RPMI buffer. This procedure was repeated after washing the cells once in the buffer. This procedure routinely eliminated 50 ± 12% of the total cells.

Rabbit anti-mouse immunoglobulin was generously donated by Drs. Richard and Catherine Crandall. The antiserum was centrifuged at 20,000 rpm for 30 minutes and the supernatant fluid was filtered through a 0.45 micron filter and the resulting effluent frozen in 1 ml aliquots. Splenocytes at a total concentration of 2 x 10^7 cells were incubated with the rabbit anti-mouse immunoglobulin (1/16) and guinea pig complement (1/10) for 1 hour at 37°C in the RPMI buffer. This procedure was repeated after washing the cells once in the buffer. This routinely eliminated 40 ± 4% of the cells.

In order to identify cell types in a population, fluorescein-conjugated antiserum was used. FITC conjugated rabbit anti-mouse IgM(1/10) (Miles Laboratories, Inc., Elkhart, IN) or FITC conjugated mouse anti-theta ascites fluid (1/10) (Litton Bionetics, Inc. Kensington, MD) was used to stain cells dried on a glass slide. The cells were suspended in the RPMI buffer at 2×10^7 per ml. Ten microliters were added to each slide and air dried. The appropriate reagent was added for 15 minutes and the slide was washed with 10 ml of the RPMI buffer. The slides were read in a Nikon Labophat microscope with an EPI fluorescence attachment (Nippon Kogaku, Inc., Garden City, NY).

Rabbit anti-mouse immunoglobulin Immunobead (Bio-Rad Laboratories, Richmond, CA) reagent (100 ml) was added to 5×10^6 cells in 1 ml of the RPMI buffer and incubated at 4°C for 1 hour. The cells were pelleted at 800 rpm for 12 minutes and the pellet was gently resuspended in 1 ml of buffer. The cells were monitored on a hemocytometer for percent rosette positive cells.

Iodination

Iodination was performed by adding 50 µl 0.2 M phosphate buffer, pH 7.2, 100 µg rabbit-anti-*A. viscosus* T14V immunoglobulin precipitated by ammonium sulfate (2 x 40%), 25 µl Enzymobead Reagent (Bio-Rad Laboratories, Richmond, CA) 1 mCi $Na^{125}I$ (New England Nuclear, Boston, MA) and 25 ml 1% Beta-glucose into a test tube. The reagents were

incubated with agitation for 25 minutes at 22°C. The solution was dialyzed extensively against saline and centrifuged at 10,000 x g for 30 minutes. The resulting supernatant fluid was stored at 4°C.

Radioimmunoassay

Cell walls were digested according to the procedure to Yokagawa et al. (81, 82). M-1-N-acetylmuramidase (M-1) (courtesy of Dr. Kanae Yokagawa, Dainippon Pharmaceutical Co., Osaka, Japan) was added to a cell wall suspension (1 mg/ml, 0.05 M Na_2HPO_4) and incubated in a 37°C water bath. Solubilization of the cell wall was monitored as the percent decrease in optical density (600 nm) at various time intervals during a 24-hour period.

Flexible, polyvinyl microtiter plates (Cooke Engineering Corp., Alexandria, VA) were incubated with the supernatant fluid of an M-1 digest of A. viscosus T14V cell walls at 2.5 µg/well in 0.1 ml, 37°C overnight. The plate was then washed with saline containing 10% fetal calf serum. The plate was washed with saline containing 15% fetal calf serum (Gibco, Grand Island, NY) (RIA buffer). The serum to be tested was diluted to the appropriate level and 0.1 ml was added per well, all sera were tested in quadruplicate at three dilutions, 22°C, 1 hour. The plate was again washed with the RIA buffer and 0.1 ml of ^{125}I-rabbit anti-T14V was added per well and incubated at 4°C overnight. The plate was then washed, dried, cut into single wells, and counted

on a Searle 100 sample gamma counter (Searle Analytic, Inc., North Miami, FL). Control wells consisting of 1) no radio-labeled antiserum, 2) radio-labeled antiserum added to uncoated walls, 3) hyperimmune mouse antisera at varying dilutions, and 4) normal mouse sera were utilized to obtain correction values for test cultures.

Histology

Mice from infected or control groups received 10 mg of Ketalar (Ketamine HCl, Parke-Davis, Detroit, MI) in .1 ml, intraperitoneally (i.p.) and 10 minutes later were decapitated and the heads stripped of fur and put into 100 ml of 4% glutaraldehyde in 0.2M cacodylate buffer in pH 7.4. After one hour, the heads were sagitally sectioned and placed in fresh buffer for 48 hours to further fix the tissue. A quadrant of each head was then dissected and placed in 5% formic acid for 7-14 days to decalcify the tissue. The tissues were dehydrated through ascending concentrations of alcohol to 100%. The tissues were infiltrated with JB-4 plastic (Polysciences, Inc., Warrington, PA) and embedded. Blocks were sectioned with glass knives on a Porter-Blum MT-1 ultramicrotome. Sections were placed on glass slides with ammonium hydroxide. Sections were stained in Ehrlich's hematoxylin and counterstained with eosin. The sections were dehydrated through alcohol and xylene.

Bone Loss Measurements

The left side of the maxilla was removed from the head of the mouse after autoclaving the head in water. The jaws were defleshed with forceps and placed in a 5% solution of Biz laundry detergent at 37°C, overnight. The jaws were then washed with distilled water and dried. Photographs were taken of the jaws with a Nikon F-2 mm Reflex camera equipped with micro-Nikkor lens and electronic ring flash and Kodak Tri-X pan film (Eastman Kodak, Rochester, NY). The perimeters of the three molars per jaw were then measured with a MOP-3 Image Analyzing System (Carl Zeiss Co., New York, NY). Bone loss was measured by comparing tooth perimeters within a group of identical animals. Bone loss is inversely related to tooth perimeters. As the bone recedes, more of the tooth surface is exposed; therefore measurement of the tooth surface exposed gives an indirect measurement of bone loss.

Radial Immunodiffusion

Rabbit anti-mouse IgG, IgM, IgA (Miles Laboratories, Elkhart, IN) was diluted 1/8, 1/16, 1/32, respectively and 200 µl of each was added to 2 ml of .75% agarose in immunoelectrophoresis buffer, 52°C. The solution was mixed and poured onto a microscope slide and allowed to solidify. Five wells were cut into each agarose slide and 10 µl of organ culture supernatant fluid was added to each well. The slides were incubated at 22°C for 2 days in a humid chamber,

dialyzed versus saline, then distilled water. They were then dried overnight and stained with Coomassie Blue. The diameters were then measured and compared to a standard curve.

Organ Culture

Gingival tissue was excised from mice that were previously sacrificed by decapitation. Tissue was removed from all four quadrants and placed in round-bottom microtiter wells. Two hundred microliters of RPMI 1640 contained 1% bovine serum albumin, 2mM HEPES, and 50 µg/ml gentamycin. Buffer was replaced each day and the spent media saved and pooled over 3 days of culture. The pools were centrifuged at 20,000 rpm, 20 minutes and the supernatant fluid was saved and tested for Ig production by radial immunodiffusion.

Chemical Extraction of Whole Cells

A modification of the method of Lancefield and Perlmann (83) was used for chemical extraction of whole cells and cell walls. Washed, freshly-cultivated whole cells (0.25 g [wet weight] per ml) or lyophilized cell walls (0.25 mg [dry weight] per ml) of A. viscosus T14V and T14AV were suspended in 0.04N HCl with 0.85% NaCl and heated in a boiling water bath for 15 minutes. Suspensions were cooled in an ice bath to room temperature and then titrated to neutrality by dropwise addition of 2N NaOH in saline. Insoluble material

was removed by centrifugation at 25,000 x g for 15 minutes. The supernatant fluid was lyophilized. Lancefield extracts of cell walls were dialyzed extensively against distilled water before lyophilization.

Preparation of Antisera

Hyperimmune sera were prepared by intravenous injections of A. viscosus antigen preparations into New Zealand white rabbits. Strain T14V cells were grown in TSBS to late exponential phase, harvested, and washed with saline. Cells were suspended at 5-6 mg (wet weight) per ml in saline and placed in a boiling water bath for 15 minutes. Four rabbits were initially injected with 0.1 ml of killed whole-cell suspensions. Beginning with week 2 and continuing at weekly intervals, rabbits were injected intravenously with 1 ml of the whole-cell suspension. Rabbits were bled from the marginal ear vein or by cardiac puncture once per week beginning 7 weeks after the initial immunization. The sera from individual rabbits were pooled before use. No immune reactions were detected between antigen preparations and serum taken before immunization.

Immunoelectrophoresis

Antigens present in the various extracts were detected by Laurell rocket immunoelectrophoresis (LRI) (84). Wells were cut approximately 1 cm from the edge of a glass slide (2 by 3 inches [ca. 5 by 7.6 cm]) containing 4 ml of 0.75%

agarose in 0.043 M sodium barbital buffer, pH 8.3. Up to 10 µg of the desired antigen was added to the wells. The agarose 2 mm above the wells was removed and replaced with 3 ml of agarose containg 10-50 µl of antiserum per ml of agarose.

In some cases, the Osserman modification (85, 86) was used as an aid in detecting antigenic identity between preparations. For this modification, a trough containing 50 µl of agarose plus 50 µl of the desired reference antigen (10 mg/ml agarose solution) was positioned 1 to 2 mm below the wells. One well containing a Lancefield extract of known composition was included as an internal standard. After electrophoresis at 8 mA/slide, gels were placed in saline overnight and then dialyzed in water for 2 hours. Gels were dried and subsequently stained with 0.5% Coomassie brilliant blue R (Sigma Chemical Co., St. Louis, Missouri) in 95% ethanol-glacial acetic acid-water (4.5:1.0:4.5, vol/vol). Destaining was carried out in the above solvent system.

Statistics

The data were analyzed using the Analysis of Variance Procedure based on the means of values within a group. Duncan's New Multiple Range Test was then used to compare the different time means for combinations of interest (87). This procedure compares each mean with every other mean by

using a set of significant ranges, each range depending upon the number of means in the comparison. The data presented represents a typical experiment. All experiments were repeated with similar results.

RESULTS

Parameters of the Mouse Periodontitis System

Infection of Conventional Balb/c Mice with A. viscosus T14V

This initial set of experiments was designed to determine if A. viscosus T14V could colonize the oral cavity of conventional Balb/c mice. Conventional Balb/c female mice were superinfected with the streptomycin resistant isolate of A. viscosus T14 (i.e. T14VJ1).

The results of a minimum infective dose curve in Balb/c mice with A. viscosus T14VJ1 are shown in Table 1. All values were from animals infected for four weeks. The colony-forming-units (CFU) recoverable differed for animals on the soft, high carbohydrate diet and those on the hard, mouse chow diet. The soft diet animals were always infected at a higher level than those on the hard diet. These results were significantly different to $p < .0001$ (student t test).

The CFU recoverable also changed with the dose of the initial inoculum. This difference was not significant within the hard diet animal's group. More CFU of A. viscosus T14VJ1 could be recovered from soft-diet-fed animals inoculated with 1.6×10^9 CFU than those inoculated with $1.6 \times$

TABLE 1

THE MINIMUM INFECTIVE DOSE IN MICE
WITH A. viscosus T14VJ1

CFU Inoculum	CFU per Molar[a]	
	Soft Diet	Hard Diet
1.6×10^9	$(13 \pm 3) \times 10^4$	900 ± 430
1.6×10^8	$(11 \pm 2) \times 10^4$	750 ± 320
1.6×10^7	$(10 \pm 2) \times 10^4$	710 ± 410
1.6×10^6	$(8 \pm 1) \times 10^4$	$<2 \times 10^2$[c]
1.6×10^5	$<2 \times 10^2$	$<2 \times 10^2$
1.6×10^4	$<2 \times 10^2$	$<2 \times 10^2$

a) each value represents the mean values of 24 molars from 4 mice

b) all mice were sacrificed at 4 weeks of infection

c) minimum level of detection

10^6CFU. The minimum infective dose of animals on a hard diet was 1.6×10^7 CFU while the minimum infective dose of soft-food-fed animals was 1.6×10^6 CFU.

Figure 1 shows the effect of time on CFU of A. viscosus T14VJ1 recoverable from mice infected with 1.6×10^7 CFU. Again, the number of CFU recoverable differed according to the diet of the animal. The soft-diet animals were colonized with greater numbers of CFU than were the hard-diet mice and both groups maintained the infection throughout the experiment.

The rate of recovery also changed when diets of the animals were compared. The CFU of the hard-diet mice remained essentially constant over 6 weeks of infection. The CFU of mice on the soft diet increased over time of infection and was still increasing at 6 weeks post-infection. Subsequent studies (see Figure 10 below) revealed maximum colonization between 6 and 8 weeks post-infection.

These data indicate that the bacteria could be recovered from the teeth of mice 6 weeks after inoculation. The recovery of bacteria was affected by the initial dose of the bacteria used for infection and the diet of the animals subsequent to infection. A soft, high carbohydrate diet fostered a higher level of oral colonization than a hard mouse-chow diet.

In order to determine whether the ability of A. viscosus T14VJ1 to colonize tooth surface changes as a function of the age of the host, colonization experiments

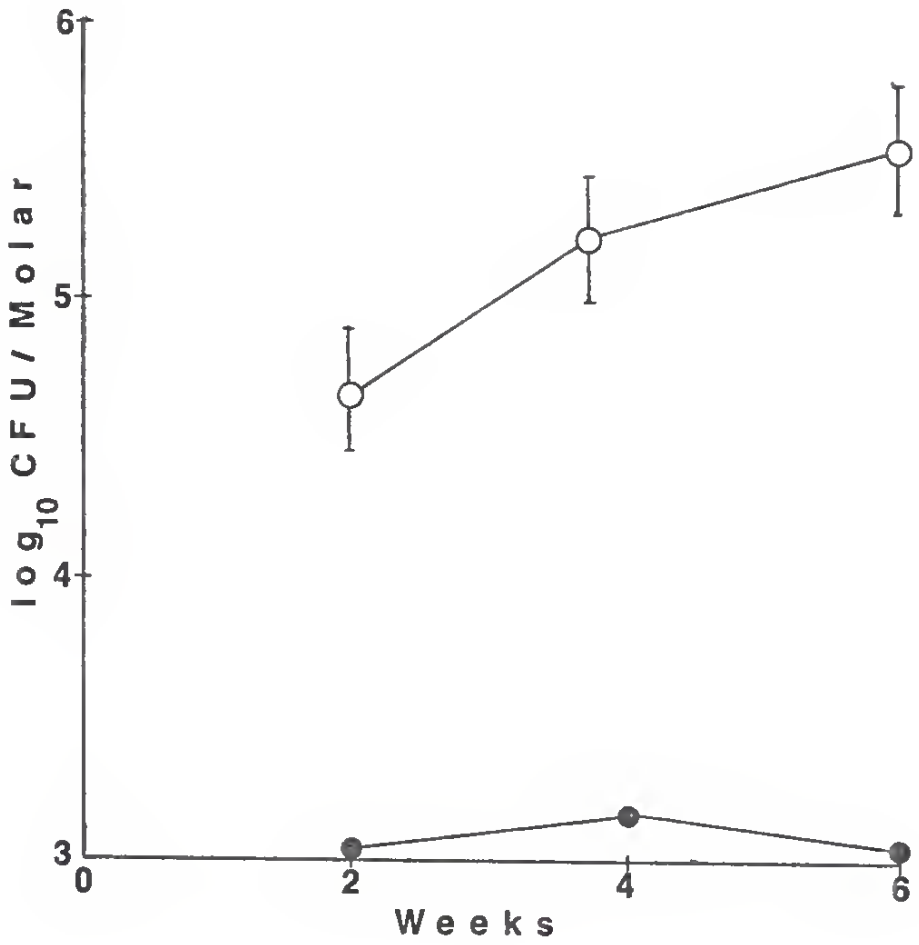

Figure 1. Colony forming units (CFU) per molar recoverable of A. viscosus T14VJ1 over 6 weeks of infection. Balb/c mice were inoculated with 1.6 x 10^7 A. viscosus T14VJ1 and fed a soft diet (open circles) or a hard diet (closed circles). Each point represents the mean value of 24 molars from 4 mice. The vertical bars represent the standard deviation.

were performed in mice of varying ages. The results of these experiments (Figure 2) revealed a distinct difference in the ability of A. viscosus cells to colonize the oral cavity of Balb/c mice of different ages. From these data, it appears that the optimal age of the oral establishment of A. viscosus T14VJ1 in these mice is 1.5 to 4 months. At any age after 6 months, it was difficult to infect the animals with A. viscosus T14VJ1. After 12 months, no infection was detected.

The results of experiments designed to determine whether bacteria inoculated into the mouths of mice remained localized in the oral cavity are shown in Table 2. The data reveal that no A. viscosus T14VJ1 cells were recoverable from sites other than the oral cavity. This effect did not change over 4 weeks of infection. When tooth samples were plated on selective medium without streptomycin, A. viscosus was not recoverable from molars of animals that were not inoculated previously. These mice do not have an indigeneous flora containing A. viscosus.

Ability of Balb/c Mice to Respond Immunologically to A. viscosus

The data in Table 3 are from experiments designed to determine whether Balb/c mice immunized intraperitoneally with A. viscosus T14V respond to the antigens. The data are a comparison of the splenic response of normal and immunized animals to A. viscosus antigens. The counts above

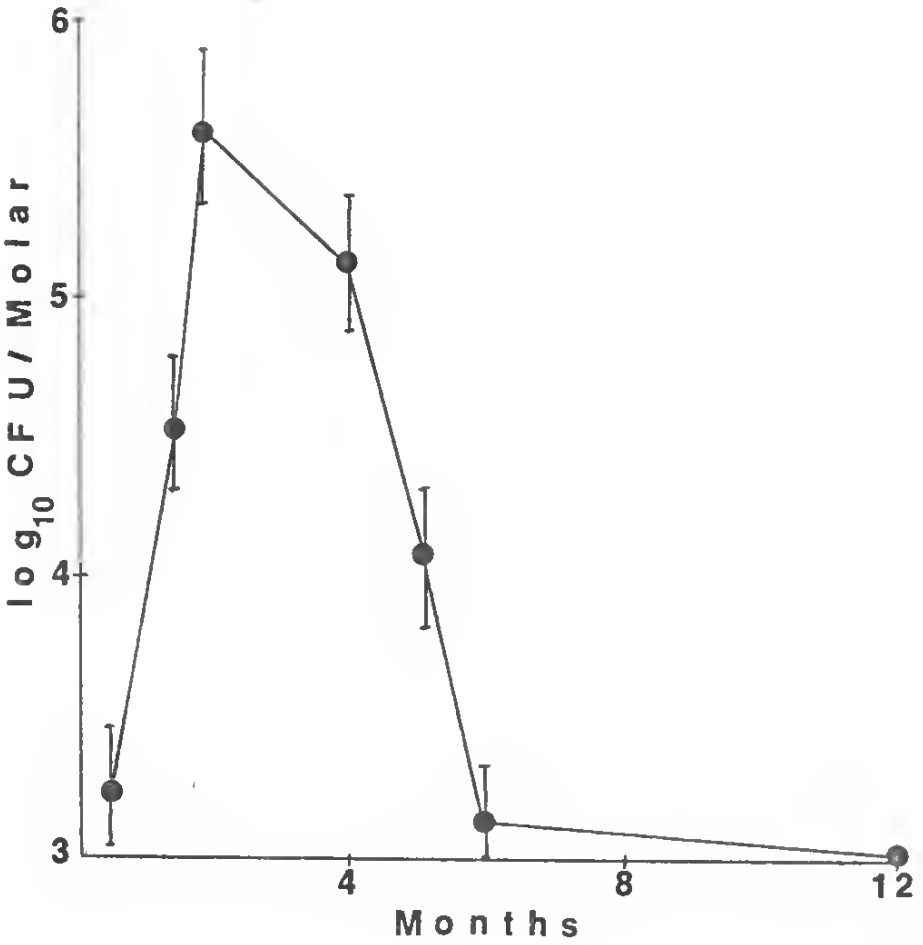

Figure 2. CFU per molar recoverable of A. viscosus T14VJ1 as a function of the age of the mouse at the time of inoculation. Mice were sacrificed at 2 weeks post-infection. Each point represents the mean of 24 molars from 4 mice. The vertical bars represent the standard deviation.

TABLE 2

ABILITY OF A. viscosus T14VJ1 TO COLONIZE VARIOUS ORGANS

Organ	Soft Diet		Hard Diet	
	2 Weeks	4 Weeks	2 Weeks	4 Weeks
Teeth[a]	50 ± 10[b]	100 ± 20	<0.2	<0.2
Others[c]	<0.2	<0.2	<0.2	<0.2

a) all values represent the mean values of 24 molars from 4 mice

b) CFU per molar x 10^3

c) includes tongue, stomach, spleen, liver, and small intestine

d) all tissues from mice inoculated with 1.6 x 10^7 CFU A. viscosus T14VJ1

TABLE 3

CAB TO A. viscosus ANTIGENS OF SPLENOCYTES OF MICE IMMUNIZED WITH A. viscosus T14V

Antigen	Conc./Well	CAB[a] Normal	CAB[a] Immunized
Sonic Walls	0.1 µg	1120 ± 150	3940 ± 320
	1.0 µg	820 ± 80	9510 ± 600
	10.0 µg	10 ± 9	18200 ± 880
Sonic Supernatant	0.1 µg	940 ± 90	5900 ± 900
	1.0 µg	3220 ± 230	11640 ± 1010
	10.0 µg	4100 ± 360	24110 ± 1120
Whole Cells	0.1 µg	4600 ± 400	8530 ± 600
	1.0 µg	5370 ± 700	9510 ± 890
	10.0 µg	840 ± 100	7540 ± 810

a) CAB = counts above background

background (CAB) for all the antigens were higher after pre-immunization with whole cells of A. viscosus T14V. This effect was seen with all antigenic preparations used.

Figure 3 shows the serum antibody levels measured by quantitative inhibition of the radioimmunoassay in animals harvested after 2, 4 and 6 weeks of immunization. A humoral response to the A. viscosus antigens was detected upon i.p. immunization with A. viscosus T14V. No serum antibody was detected in animals not previously immunized with A viscosus T14V. These data suggest that the normal mouse is not making detectable antibody to an antigen of A. viscosus T14V nor to a cross-reacting antigen.

Bone Loss in Animals Infected with A. viscosus T14VJ1

The next set of experiments was designed to determine whether the colonization of A. viscosus T14VJ1 cells on the teeth of Balb/c mice resulted in the initiation of a sequence of events leading to bone resorption and subsequent tooth loss. The data in Figure 4 show the results of a 3-month oral infection of Balb/c mice with A. viscosus T14VJ1. The second maxillary molars were chosen because they were present in all the jaws tested. Many of the other molars were missing from the jaws of infected animals. Although not all infected mice in these studies lost teeth, the examination of a number of orally infected mice reveals a strong, positive correlation between the loss of bone structure and tooth loss. Animals on a soft diet attained

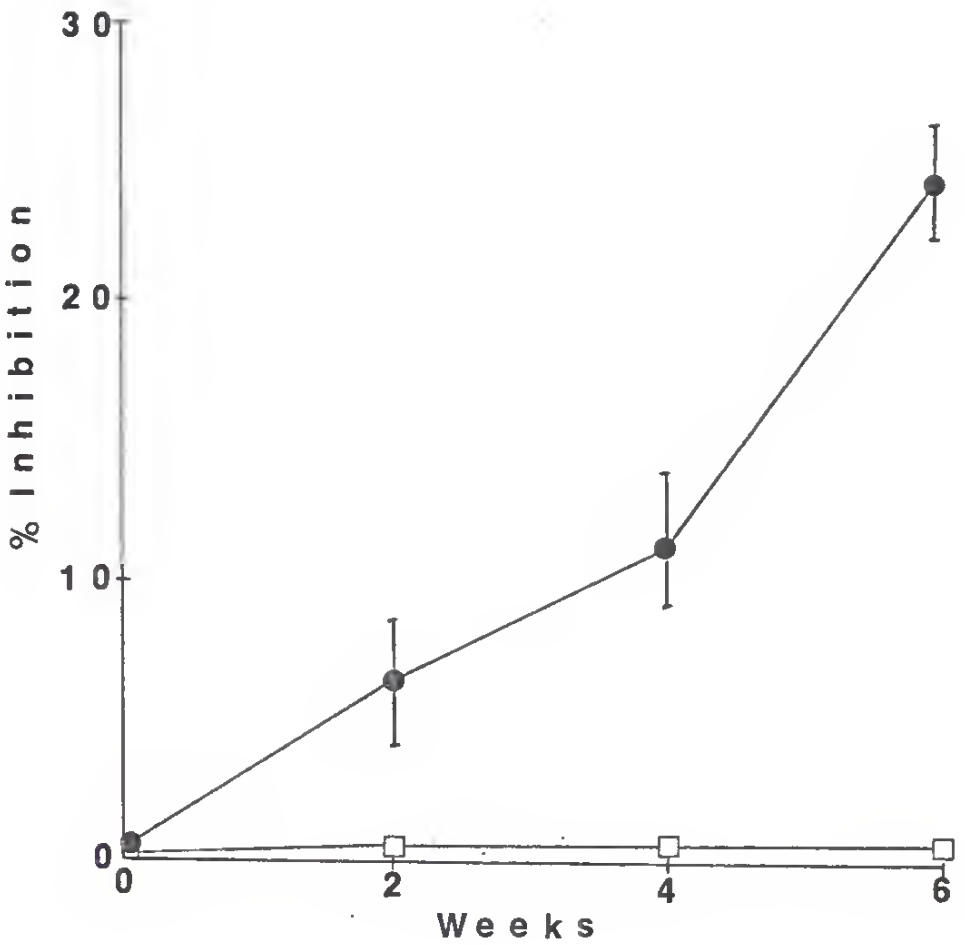

Figure 3. The ability of Balb/c mice to produce an antibody response to A. viscosus T14V, as detected by RIA. Animals were immunized i.p. with 100 µg A. viscosus T14V at 2-week intervals (closed circles) or sham immunized. Each point represents the mean value of 16 samples from 4 mice. The vertical bars represent the standard deviation.

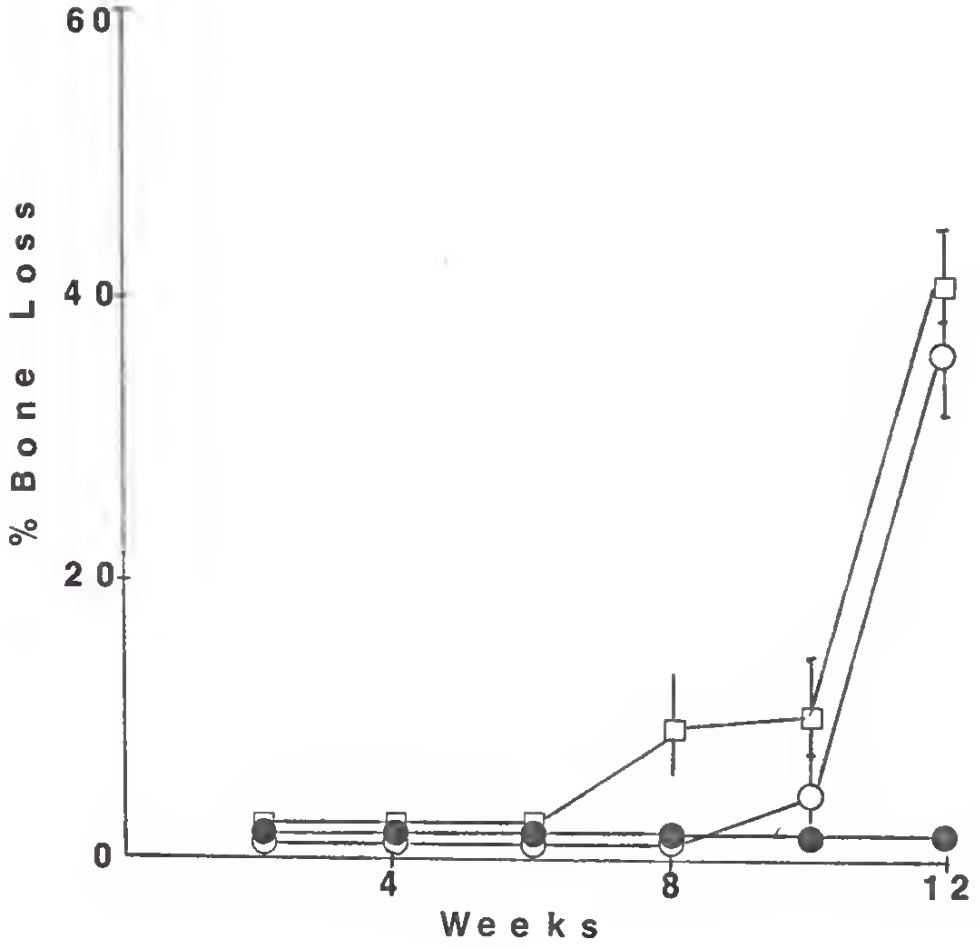

Figure 4. The ability of A. viscosus T14VJ1 to induce bone loss in the maxillae of Balb/c mice after 12 weeks of infection. Mice on a soft diet (open squares) or on a hard diet (open circles) were inoculated with 1.6×10^7 CFU. Control mice were fed either a hard or soft diet (closed circles). Tooth perimeters were measured on the second maxillary molars. Each point represents 8 molars from 4 mice. The vertical bars represent the standard deviation.

a more pronounced bone loss than hard-diet animals but the hard-diet animal's bone loss was evident. The jaws in Figure 5 are typical examples from infected mice.

Analysis of the Immune Response in Orally Infected Animals on a Hard Diet

Mice, 6-8 weeks old, were inoculated orally with 1.6×10^9, 1.6×10^8 or 1.6×10^7 CFU of A. viscosus T14VJ1 and placed on a hard diet for three months. At two-week intervals, three animals per group were sacrificed and the spleens were removed and analyzed by lymphoblast assay with antigens of A. viscosus T14V, Concanavalin A (ConA) or Lipopolysaccharide (LPS). The animals were bled and the sera were analyzed, using the RIA previously mentioned, for antibody to A. viscosus T14V. The jaws were sampled for CFU of A. viscosus T14VJ1 by tooth grinding, or the jaws were then defleshed by autoclaving and the tooth perimeter exposed was measured for bone loss.

Infection of Balb/c Mice with A. viscosus T14VJ1

The recoverable CFU are presented as a function of time post-infection in Figure 6. It is evident from the data that the level of infection is very low and no statistically significant difference in colonization was observed in the animals with varying doses. A point that is not obvious from the graph is that the standard deviations were very high (50-85% of the mean) and no significant numbers could be determined.

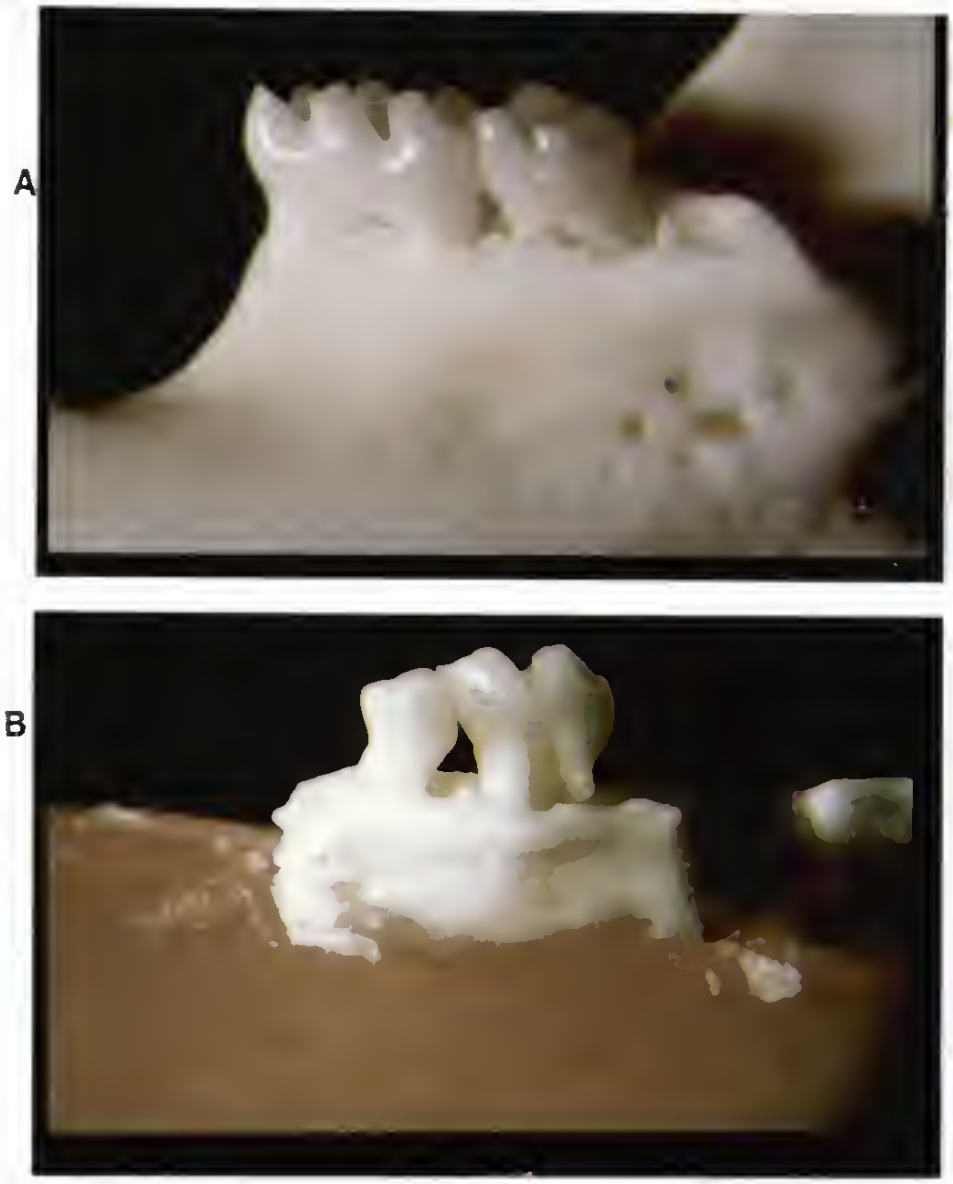

Figure 5. Typical examples of bone loss in infected. A defleshed jaw from a 12-week infected mouse on a hard diet (A) and a soft diet (B).

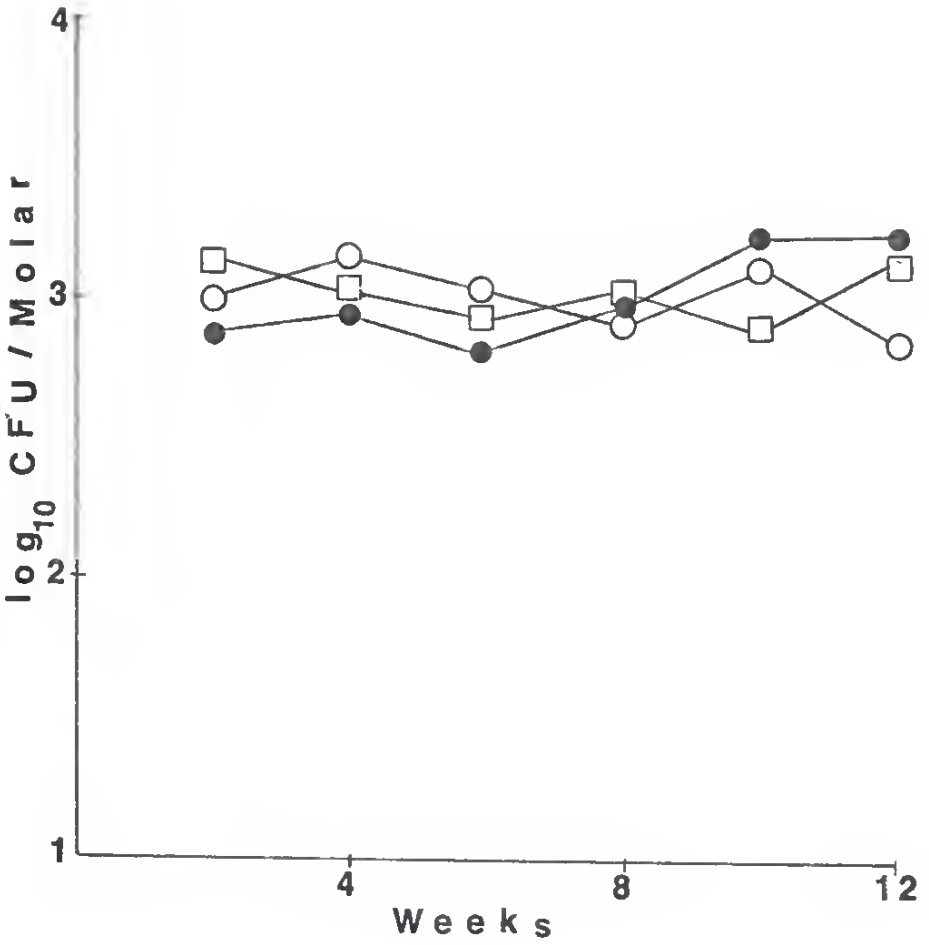

Figure 6. CFU recoverable of A. viscosus T14VJ1 in mice on a hard diet over 12 weeks of infection. Mice were inoculated with 1.6 x 10^9 CFU (closed circles), 1.6 x 10^8 CFU (open squares) or 1.6 x 10^7 CFU (open circles). Each point represents the mean value of 18 molars from 3 mice.

Cell-Mediated Responses of Infected Mice

The counts above background (CAB) are presented as a function of time post-infection in Figure 7. Mice were sacrificed at 6 biweekly intervals and the splenocytes were incubated with 12.5 µg of A. viscosus sonic supernatant fluid. This dose represents the optimum when the antigen is titrated in the lymphoblast assay. The only points that show a response different from normal, uninfected animals were the animals receiving the lowest dose of inoculum and these animals responded at all doses of the antigen at 6 weeks to 8 weeks post-infection. This response dropped to normal levels at 10 and 12 weeks. These data show a strong correlation between dose of inoculum and responsiveness. Though A. viscosus T14VJ1 was recovered from all the inoculated animals, only the low dose inoculum animals showed a splenic lymphoblast response to the antigens of the bacteria. At no time did the high-dose inoculated (1.6×10^8, 1.6×10^9 CFU) animals respond to the A. viscosus antigens above control, baseline levels. The LPS response in those animals (Figure 8) showed the same pattern as did the bacterial antigens. The LPS dose represents the optimal mitogenic concentration when the mitogen is titrated (data not shown). The only responding animals above the uninfected animal levels were those animals inoculated with the lowest dose of bacteria. The kinetics was identical to that seen with the bacterial antigens. The response increased in six- and eight-week infected animals and decreased to baseline levels

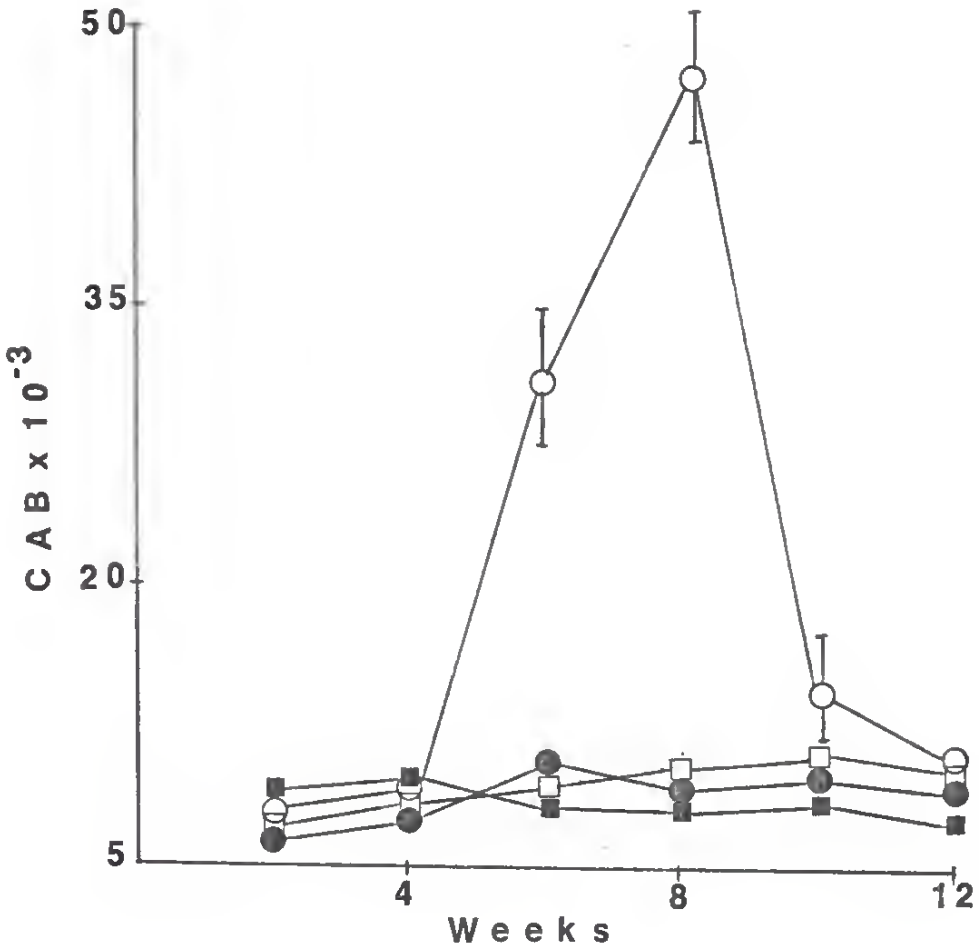

Figure 7. The splenic lymphoblast response to A. viscosus T14V sonic supernatant antigens. Mice were inoculated with 1.6 x 10^9 CFU (closed circles), 1.6 x 10^8 CFU (open squares) or 1.6 x 10^7 CFU (open circles). Uninfected mice were also maintained (closed squares). Each point represents the mean value of 12 samples from 3 mice. The vertical bars represent the standard deviation.

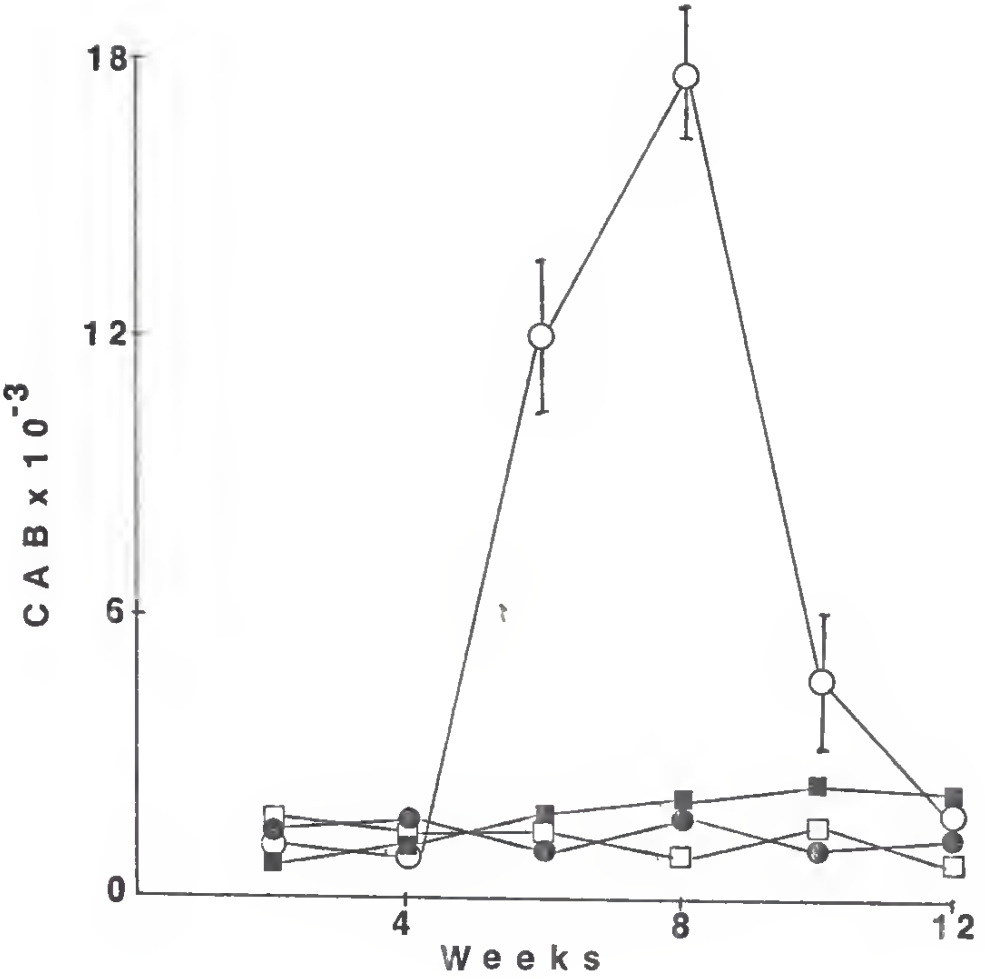

Figure 8. The splenic lymphoblast response to LPS as a function of time of infection. Mice were inoculated with 1.6 x 10^9 CFU (closed circles), 1.6 x 10^8 CFU (open squares), 1.6 x 10^7 CFU (open circles) of A. viscosus T14VJ1 or were uninfected (closed squares). Each point represents the mean value of 12 samples from 3 mice. The vertical bars represent the standard deviation.

in the tenth and twelfth week of infection. These data suggest that the initial dose of inoculum can alter the response of the animals to the bacteria as measured by the lymphoblast system. Con A reactivity remained the same in infected and uninfected animals (data not shown).

Humoral Response of Infected Mice

Figure 9 presents the serum antibody levels to A. viscosus T14V over three months of infection in the same animals as those examined in the lymphoblast assay. Antibody was detected in low dose infected animals from 6 weeks to 12 weeks. No antibody was detected in uninfected nor in high dose animal's sera.

Bone Loss in Infected Mice

When the jaws were examined for bone loss (Figure 10), the only jaws to lose bone above control levels were those belonging to the animals inoculated with 1.6×10^7 CFU of A. viscosus T14VJ1. These animals had evident bone loss at 10 and 12 weeks post-infection. Typical defleshed mouse jaws are shown in Figure 11. Though the other inoculated animals were infected, no bone loss was evident. These data suggest the infection alone is not sufficient for bone loss.

The major drawback to the previous experiments was that the level of infection in inoculated animals was variable. This does not allow statistically significant data on infection from these animals to be attained. A soft,

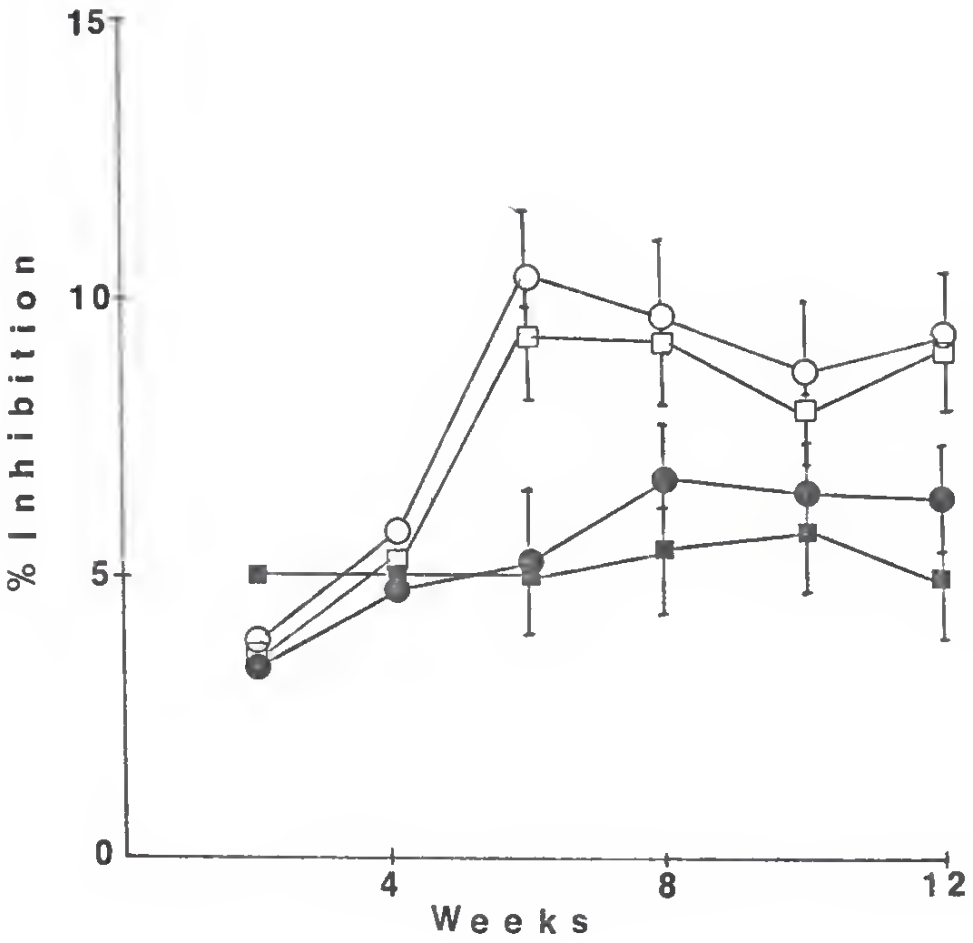

Figure 9. The serum levels of antibody to A. viscosus T14V as a function of time of infection. Mice were inoculated with 1.6 x 10^9 CFU (closed circles), 1.6 x 10^8 CFU (open squares), 1.6 x 10^7 CFU (open circles) of A. viscosus T14VJ1 or were uninfected (closed squares). Each point represents the mean value of 12 samples from 3 mice. The vertical bars represent the standard deviation.

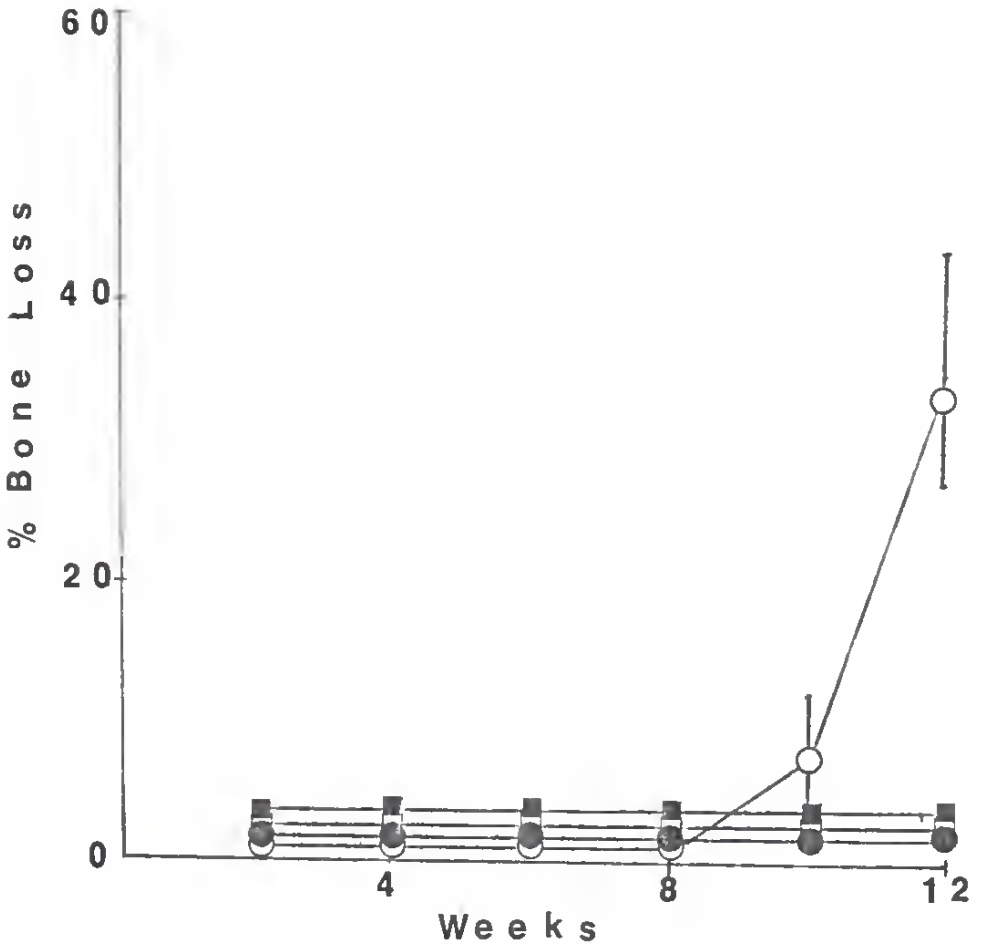

Figure 10. Percent bone loss of the second maxillary molars as a function of time of infection with A. viscosus T14VJ1. Mice were inoculated with 1.6 x 10^9 CFU (closed circles), 1.6 x 10^8 CFU (open squares), or 1.6 x 10^7 CFU (open circles). Uninfected mice were also maintained (closed squares). Each point represents the mean value of 8 samples from 4 mice. The vertical bars represent the standard deviation.

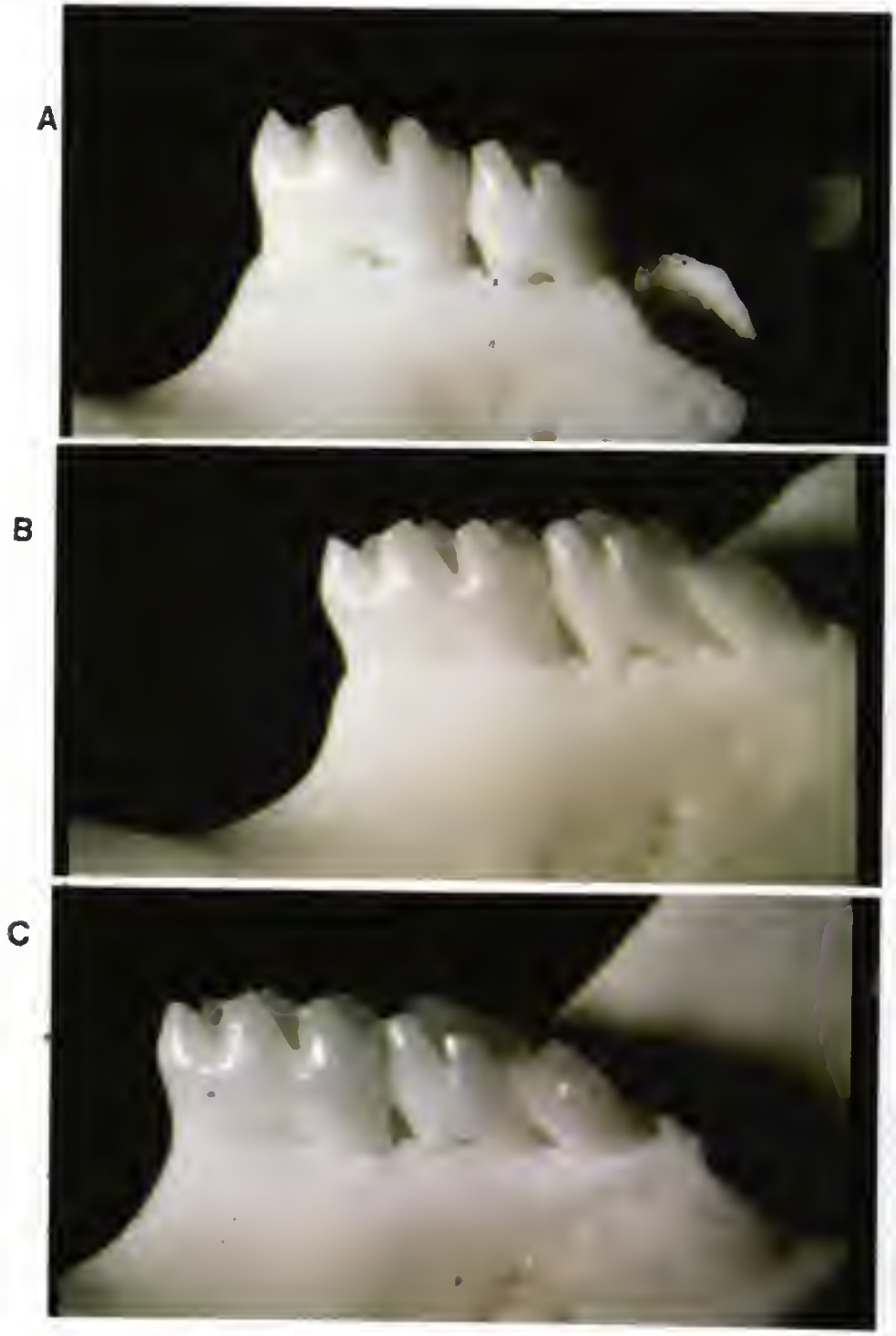

Figure 11. Typical examples of bone loss induced in infected mice on a hard diet. Defleshed jaws are from 12-week infected mice. The initial inoculum was 1.6×10^7 CFU (A), 1.6×10^9 CFU (B) or were uninoculated (C).

high-carbohydrate diet allows the bacteria to colonize in larger numbers and the level of infection is reproducible. The soft diet was used in subsequent experiments.

Analysis of the Immune Response in Orally Infected Animals on a Soft, High-Carbohydrate Diet

Animals were infected with the two minimum doses of A. viscosus T14VJ1 (i.e. 1.6×10^6, 1.6×10^7 CFU) and the high dose (i.e. 1.6×10^9 CFU) that rendered the animals unresponsive above. The lymphoblast response to A. viscosus T14V antigens, Con A and LPS were monitored over three months post-infection. Serum antibody levels, CFU per molar and bone loss were also measured.

Infection of Balb/c Mice With A. viscosus T14VJ1

The animals on a soft diet attained a much higher level of infection than the animals on a hard diet (Figure 12). The uninfected animals showed no infection (data not shown). The numbers of bacteria in soft-diet animals rose dramatically for the first month of infection reaching a plateau at 6 weeks. The level of infection was similar regardless of the initial inoculum. In hard-diet animals, on the other hand, bacteria were at a much lower level and this level differed from mouse to mouse.

To insure that the colonies that were cultured from the mice were the same that were put in, random samples of

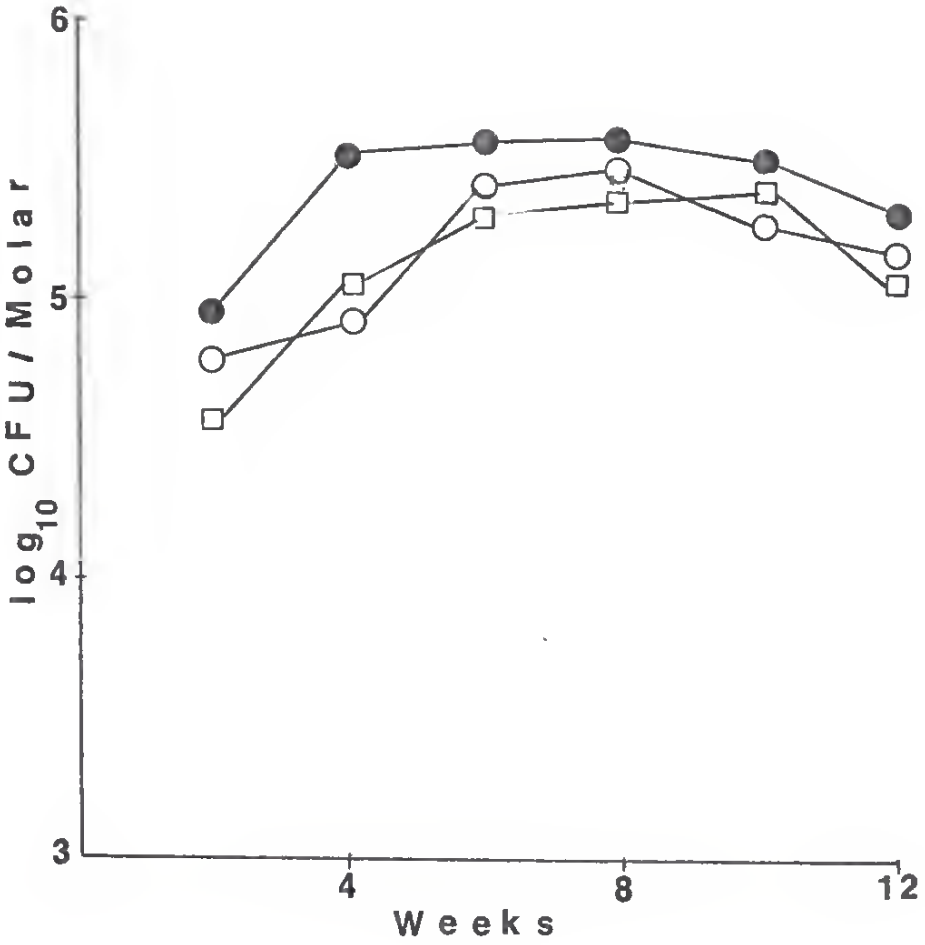

Figure 12. CFU recoverable of A. viscosus T14VJ1 in mice on a soft diet over 12 weeks of infection. Mice were inoculated with 1.6 x 10^9 CFU (closed circles), 1.6 x 10^7 CFU (open circles), or 1.6 x 10^6 CFU (open squares) of A. viscosus T14VJ1. Each point represents the mean value of 18 molars from 3 mice.

colonies from each week were grown in TSBS and extracted by the Lancefield procedure and analyzed by rocketed immunoelectrophoresis and compared to the original inoculum. These results (Figure 13) revealed that the samples recovered from infected animals were identical antigenically to the original inoculum. The four characteristic antigens in the initial inoculum were present in all the samples recovered from the infected animals.

Cell Mediated Responses of Infected Mice

In the lymphoblast response to A. viscosus antigens (Figure 14), a significant splenic response was seen in low-dose infected animals when compared to uninfected animals. This response was very much higher in both of the low-dose levels of soft-diet mice, but was never seen in the high-dose inoculated animals. The splenic lymphoblast response of the high-dose inoculated mice never differed from that seen in uninfected animals. The LPS response (Figure 15) in these same animals increased over baseline, uninfected mice levels at the same time. The LPS response mimics that seen with A. viscosus T14V sonic supernatant antigens in the lymphoblast assay, though the LPS response is a much more profound response. The Con A response did not vary between infected and uninfected animals (data not shown).

When splenocytes from these animals were fractionated on a nylon wool column, by the method of Julius et al. (80),

Figure 13. Comparison of Lancefield extracted (LE) samples of A. viscosus T14VJ1 from infected mice. The trough below the wells contained a standard Lancefield extract of A. viscosus T14V. 1) Standard LE of A. viscosus T14V. 2) LE of initial inoculum. 3) LE of 2-week sample. 4) Standard LE of A. viscosus T14V. 5) LE of 4-week sample. 6) LE of 6-week sample. 7) LE of 8-week sample. 8) Standard LE of A. viscosus T14V. 9) LE of 10-week sample. 10) LE of 12-week sample. 11) LE of initial inoculum.

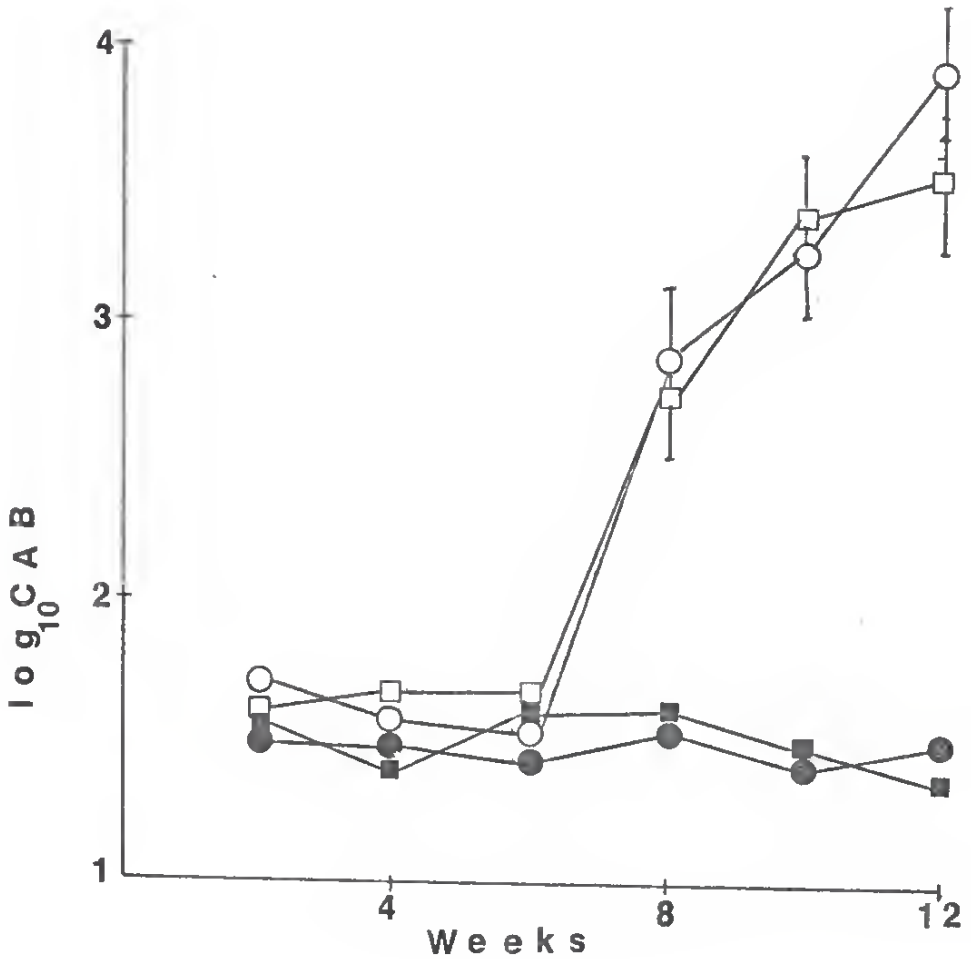

Figure 14. The splenic lymphoblast response to A. viscosus T14V sonic supernatant antigens. Mice were inoculated with 1.6 X 10^9 CFU (closed circles), 1.6 x 10^7 CFU (open circles), 1.6 x 10^6 CFU (open squares) of A. viscosus T14VJ1 or were uninfected (closed squares). Each point represents the mean value of 16 samples from 4 mice. The vertical bars represent the standard deviation.

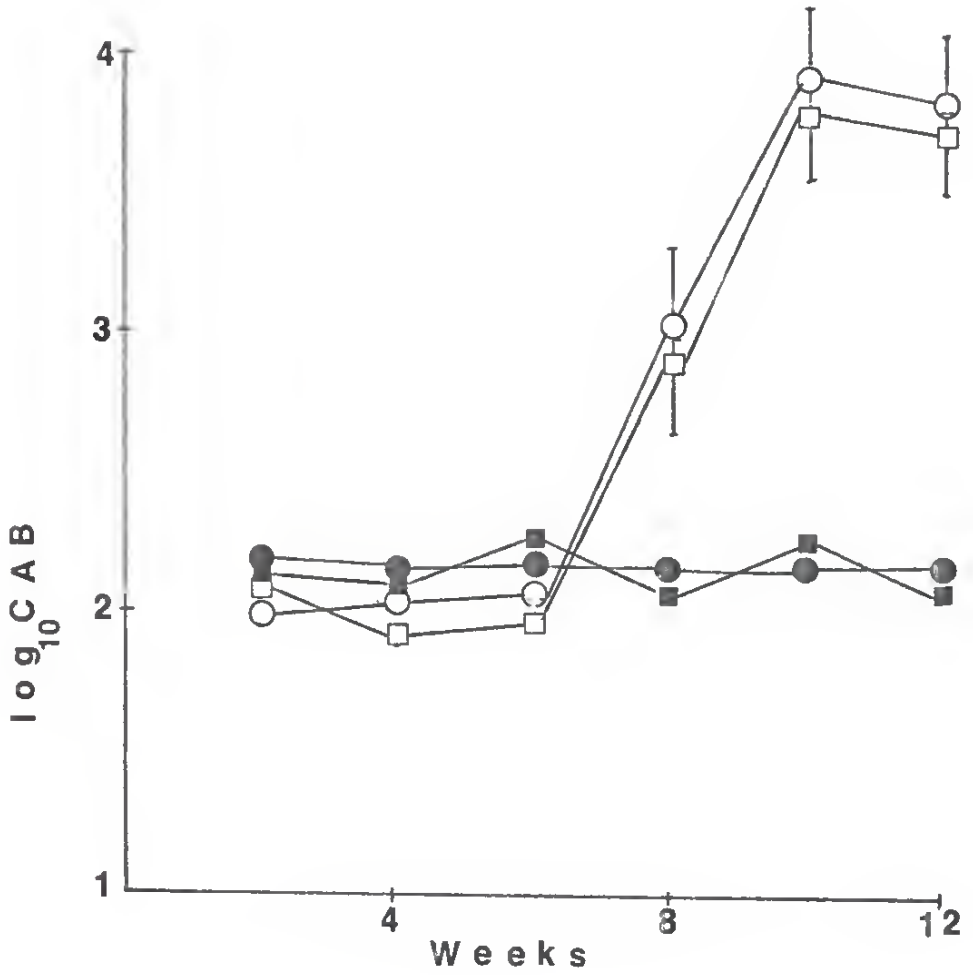

Figure 15. The splenic lymphoblast response to LPS as a function of time of infection. Mice were inoculated with 1.6 X 10^9 CFU (closed circles), 1.6 x 10^7 CFU (open circles), 1.6 x 10^6 CFU (open squares) of A. viscosus T14VJ1 or were uninfected (closed squares). Each point represents the mean value of 16 samples from 4 mice. The vertical bars represent the standard deviation.

a population was obtained that was 85% T cells, 5% immunoglobulin bearing cells and 10% other cell types. T cells were identified by fluorescein conjugated anti-theta antiserum and immunoglobulin bearing cells were identified by Bio-Rad anti-mouse immunoglobulin immunobeads. When this population was added to the lymphoblast assay (Figure 16), the cells were unresponsive to A. viscosus antigens or LPS but responsive to Con A.

More direct evidence is seen in Figure 17 and Table 4. T cells were eliminated with anti-theta serum and complement and the resulting population incubated in the lymphoblast assay with A. viscosus sonic supernatant. This population of cells was 92% B cells as judged by Immunobead anti-mouse IgG. These cells did not respond to Con A. The data show the two low-dose infected groups still responded to the A. viscosus antigens. In contrast, the high-dose infected group did not respond to the A. viscosus antigens.

This effect could be abrogated by treating these cells with an anti-mouse IgG and complement (Table 4). This population of cells had less than 10% IgG positive cells and did not respond to LPS. These data prove that the responding cell population is a B cell predominant one.

When the cervical lymph nodes from infected animals were analyzed in the lymphoblast assay for reactivity to A. viscosus sonic supernatant and LPS, a pattern similar to the splenocytes was seen. The CAB are presented as a function of time post-infection in Figure 18. An increase in

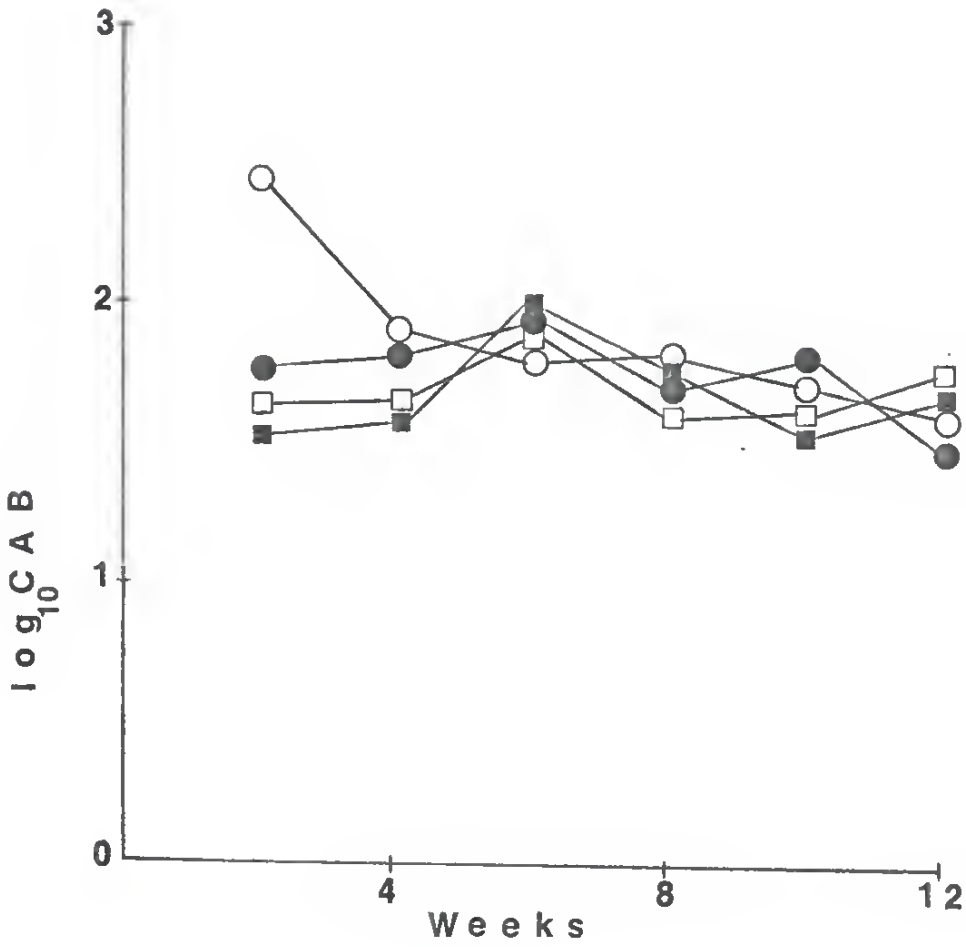

Figure 16. The splenic T cell lymphoblast response to A. viscosus T14V sonic supernatant antigens. $\overline{T}$ cells were separated on nylon wool columns. Mice were inoculated with 1.6 x 10^9 CFU (closed circles), 1.6 x 10^7 CFU (open circles), 1.6 x 10^6 CFU (open squares) of A. viscosus T14VJ1 or were uninfected (closed squares). Each point represents the mean value of 16 samples from 4 mice.

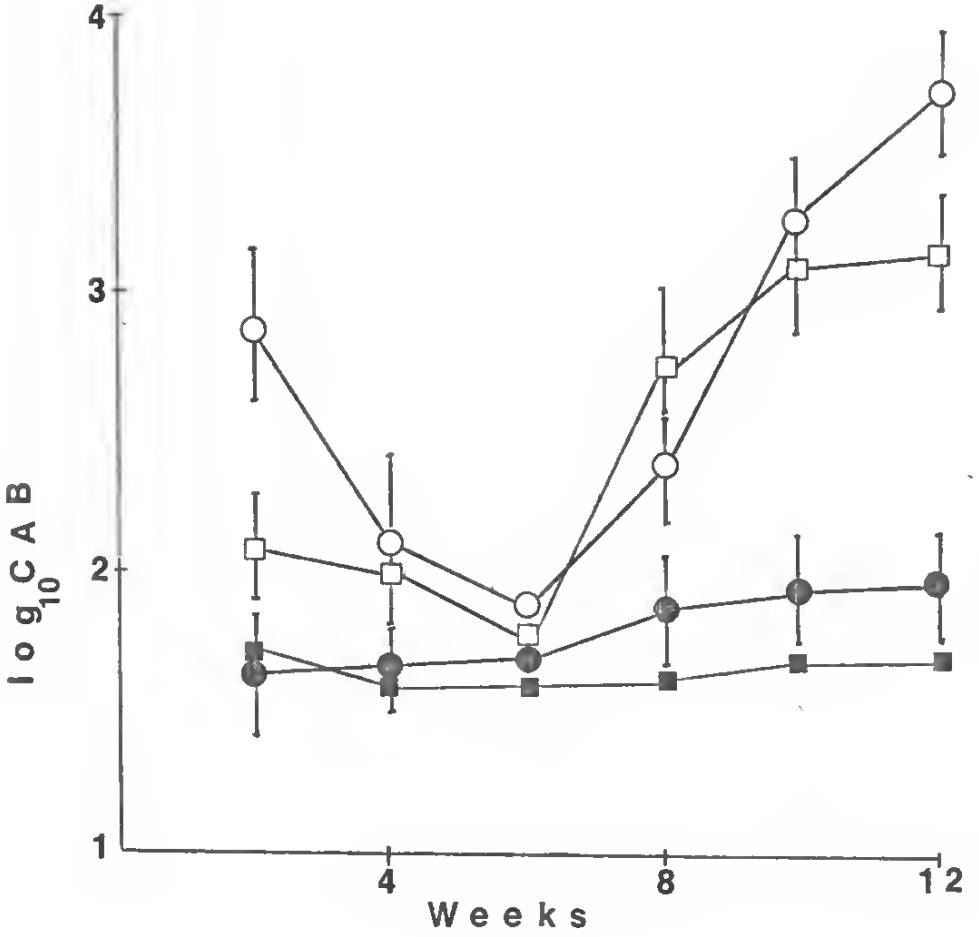

Figure 17. The splenic B cell lymphoblast response to A. viscosus T14V sonic supernatant antigens. $\overline{B}$ cells were separated by anti-theta and complement treatment of splenocytes. Mice were inoculated with 1.6×10^9 CFU (closed circles), 1.6×10^7 CFU (open circles), 1.6×10^6 CFU (open squares) of A. viscosus T14VJ1 or were uninfected (closed squares). Each point represents the mean value of 16 samples from 4 mice. The vertical bars represent the standard deviation.

TABLE 4

CELL SEPARATION OF SPLENOCYTES FROM LOW-DOSE, 12-WEEK INFECTED MICE

	Untreated	Anti-Theta + C' Treatment	Anti-Ig + C' Treatment
Total Cells	1.7×10^8	7.7×10^7	1.5×10^7
% Theta Positive	43 ± 6	3 ± 2	4 ± 2
% Ig Positive	39 ± 7	92 ± 5	8 ± 3
ConA Response	70,800 ± 1560[a]	120 ± 15	155 ± 25
LPS Response	7100 ± 870	9200 ± 960	180 ± 45
A. viscosus Sonic Supernatant	2500 ± 450	3200 ± 750	130 ± 30
Background	140 ± 35	100 ± 35	170 ± 40

a) CAB

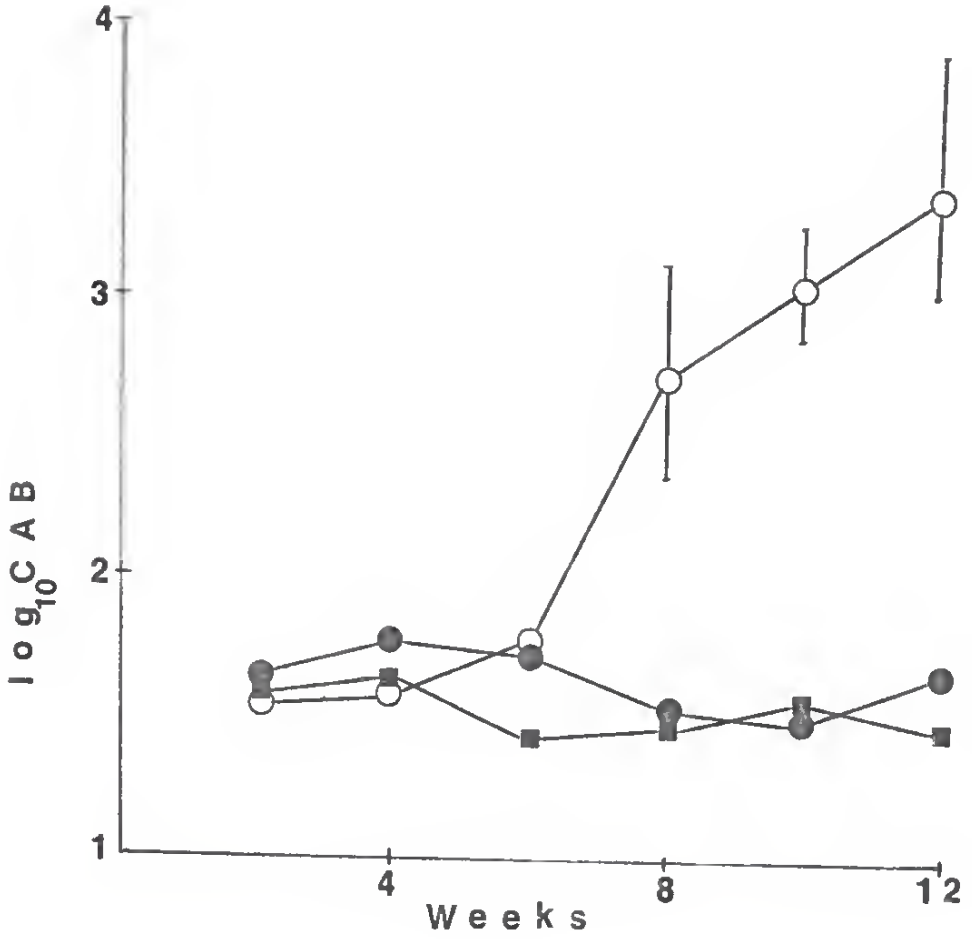

Figure 18. The cervical lymph node lymphoblast response to A. viscosus T14V sonic supernatant antigens. Mice were inoculated with 1.6 x 10^9 CFU (closed circles), 1.6 x 10^7 CFU (open circles) or were uninfected (closed squares). Each point represents the mean value of 16 samples from 4 mice. The vertical bars represent the standard deviation.

response over control levels could be seen at 8 weeks and this increase continued to 12 weeks post-infection with the antigens in A. viscosus sonic supernatant. Lymph node cells from the control or high-dose infected animals never responded to the A. viscosus antigens. The same effect is seen in response to LPS (Figure 19).

Humoral Response of Infected Mice

As described above, serum antibody levels (Figure 20) did not increase significantly above baseline levels in any of the colonized animals. Neither the dose of the inoculum nor the level of colonization altered the serum antibody levels in these mice.

Bone Loss in Infected Mice

When these animals were checked for bone loss (Figure 21), the animals with the low-dose inoculum attained an extensive level of bone loss and the levels all were significantly different than the uninfected animals. Figure 22 shows typical examples of defleshed jaws from infected mice. These data suggest that the level of oral infection affects the splenic response to the bacteria and also affects the rate of bone loss. The high-dose inoculated animals did not attain tooth perimeter measurements that were statistically different from the uninfected animals. These data extend the observations seen in the hard-food fed animals and add

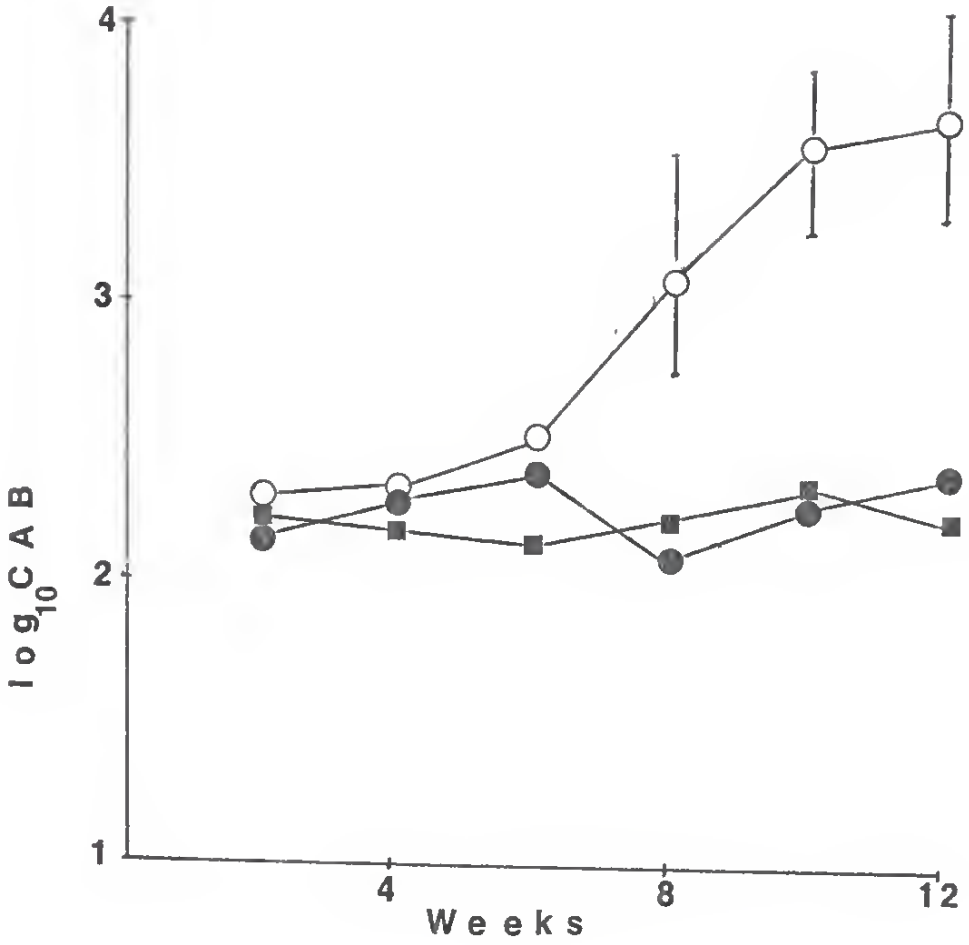

Figure 19. The cervical lymph node lymphoblast response to LPS. Mice were inoculated with 1.6 x 10^9 CFU (closed circles), 1.6 x 10^7 CFU (open circles) or were uninfected (closed squares). Each point represents the mean value of 16 samples from 4 mice. The vertical bars represent the standard deviation.

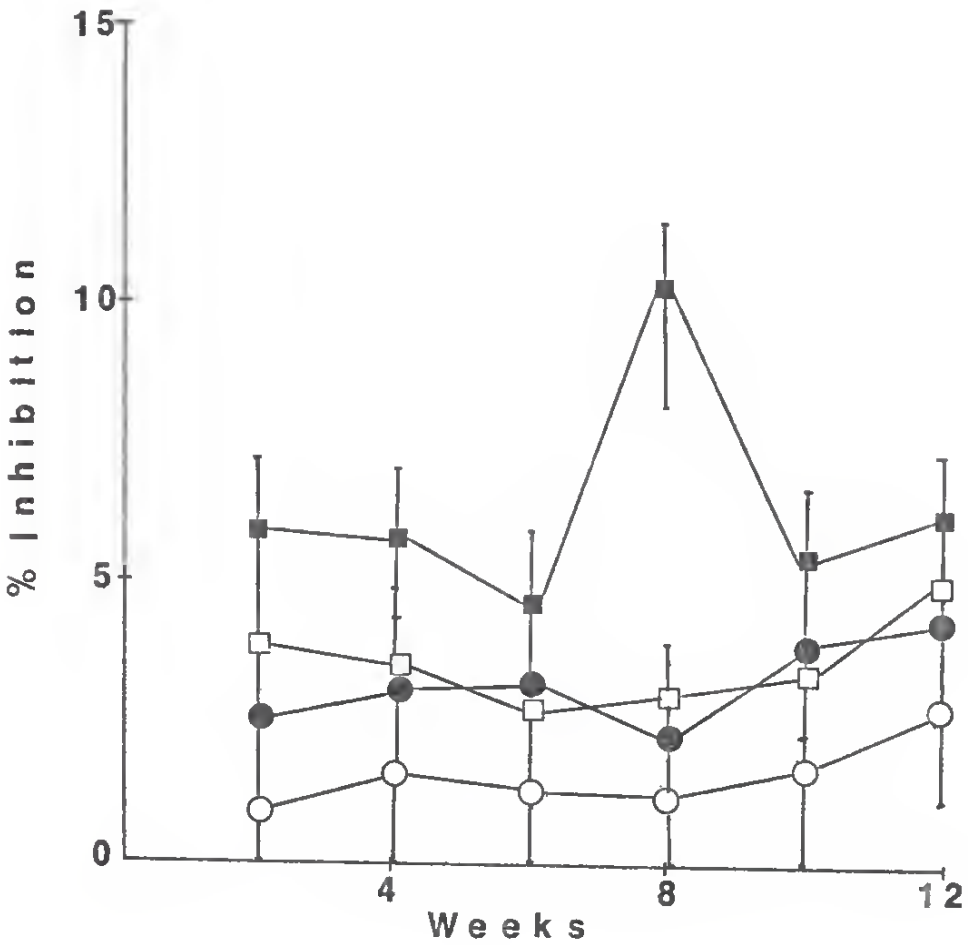

Figure 20. The serum levels of antibody to A. viscosus T14V as a function of time of infection. Mice were inoculated with 1.6×10^9 CFU (closed circles), 1.6×10^7 CFU (open circles), 1.6×10^6 CFU (open squares) of A. viscosus T 14VJ1 or were uninfected (closed squares). Each point represents the mean value of 16 samples from 4 mice. The vertical bars represent the standard deviation.

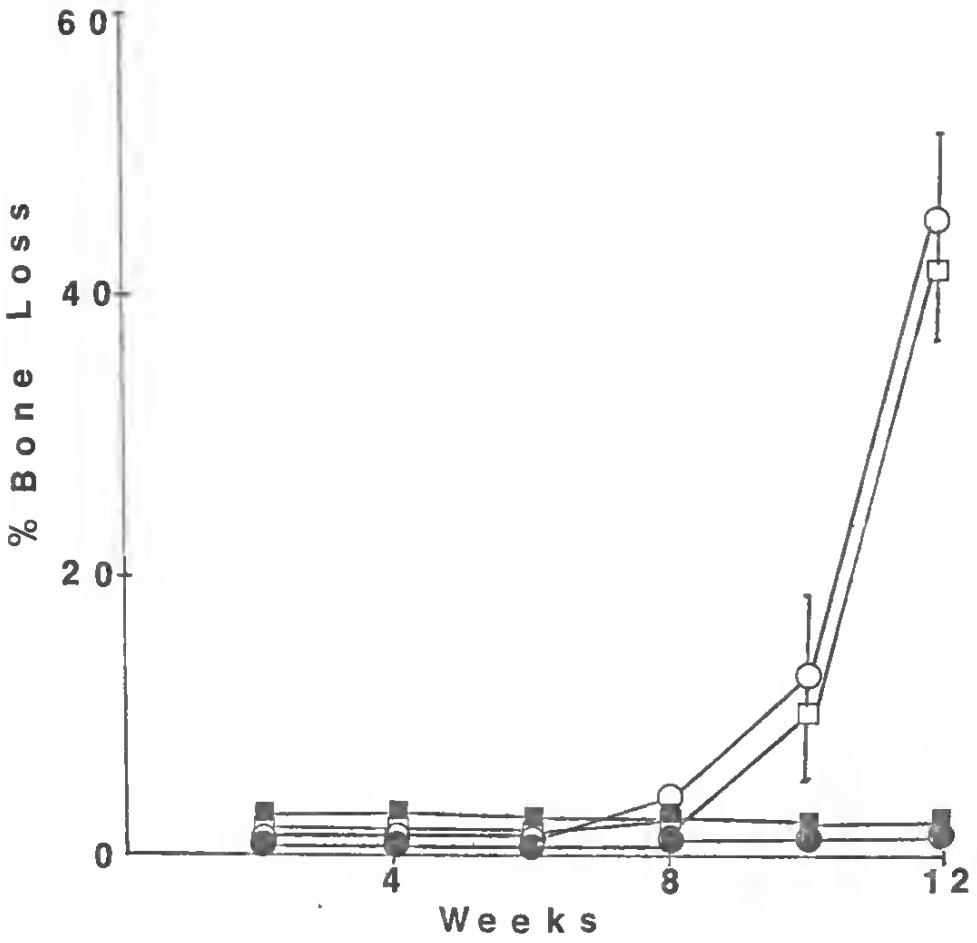

Figure 21. Percent bone loss of the second maxillary molars as a function of time of infection with A. viscosus T14VJ1. Mice were inoculated with 1.6×10^9 CFU (closed circles), 1.6×10^7 CFU (open circles), 1.6×10^6 CFU (open squares) of A. viscosus T14VJ1 or were uninfected (closed squares). Each point represents the mean value of 8 samples from 4 mice. The vertical bars represent the standard deviation.

Figure 22. Typical examples of bone loss induced in infected mice on a hard diet. Defleshed jaws are from 12-week infected mice. The initial inoculum was 1.6×10^7 CFU (A), 1.6×10^9 CFU (B) or were uninoculated (C).

the extra dimension of a stable bacterial cell count in the infected animals.

Analysis of Gingival Tissues of Infected Mice

Mice were infected with 1.6×10^9 or 1.6×10^7 CFU of A. viscosus T14VJ1 and placed on the soft-food, high-carbohydrate diet. At two-week interals, the gingival tissue was excised and placed into tissue culture. The tissue culture supernatant was then analyzed for immunoglobulin by radial immunodiffusion (RID) with the appropriate antisera. Figure 23 shows the standard curves for the RID. The assay was repeatable and sensitive to nanogram levels. When culture samples were tested in the RID assay no increase in immunoglobulin was detected in any tissue culture supernatants from infected mice.

Histology of Gingival Tissues of Infected Mice

The micrographs in Figures 24, 25, 26, 27, 28, and 29 are typical sections taken from a sagital slice of the intact jaw of normal and infected mice. The sections shown here were from 13-week infected animals or age-matched controls. The normal tissue in animals on either diet was relatively infiltrate free. The crevicular epithelium was clearly separate from the gingival tissue and all the soft tissue was surrounded by a keratinized sheath of cells.

In infected animals on either diet, the crevicular epithelium and underlying connective tissue was infiltrated

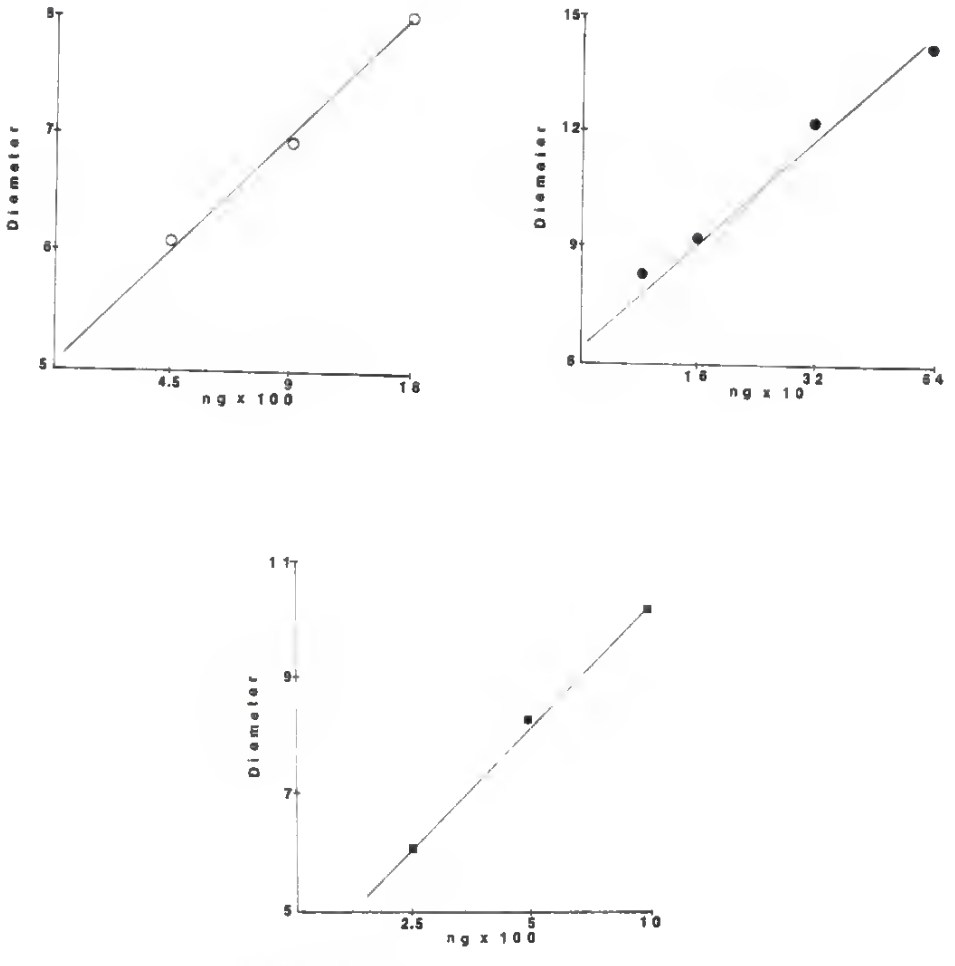

Figure 23. The standard curves used in the RID. IgA (open circles), IgG (closed circles), IgM (closed squares).

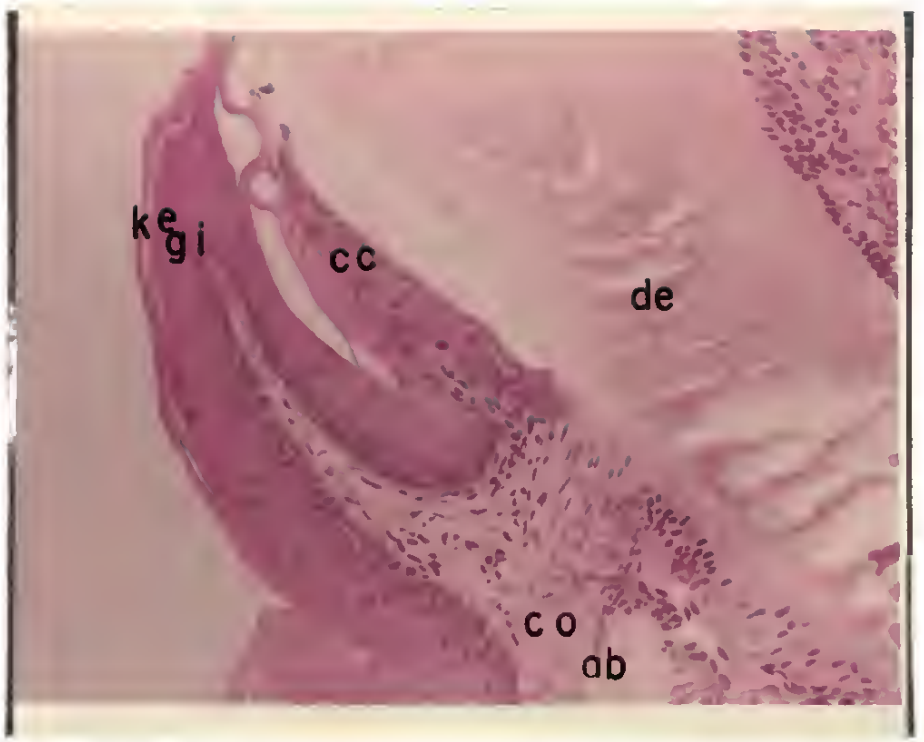

Figure 24. A typical section from a sagitally sectioned mouse jaw. This is a low magnification print showing the characteristics of the tissue: alveolar bone (ab), crevicular epithelium (cc), connective tissue (co), dentin (de), gingiva (gi), keratinized epithelium (ke). The box represents the area shown in subsequent pictures.

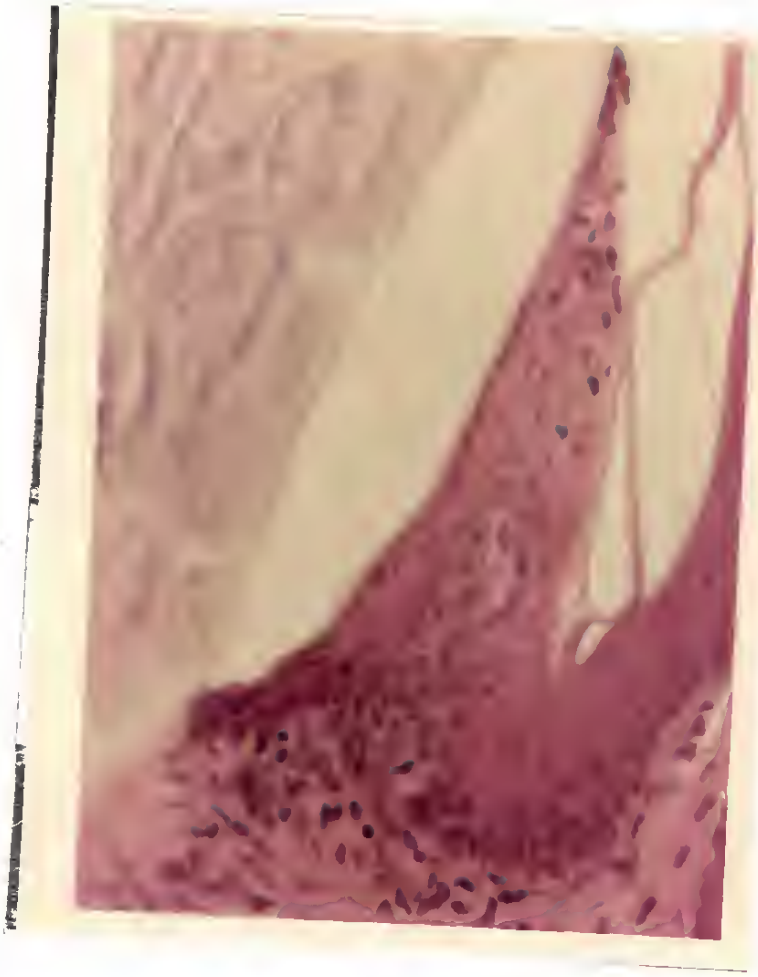

Figure 25. A typical sagital section from uninfected mice on a hard diet. These animals are thirteen weeks post-infection.

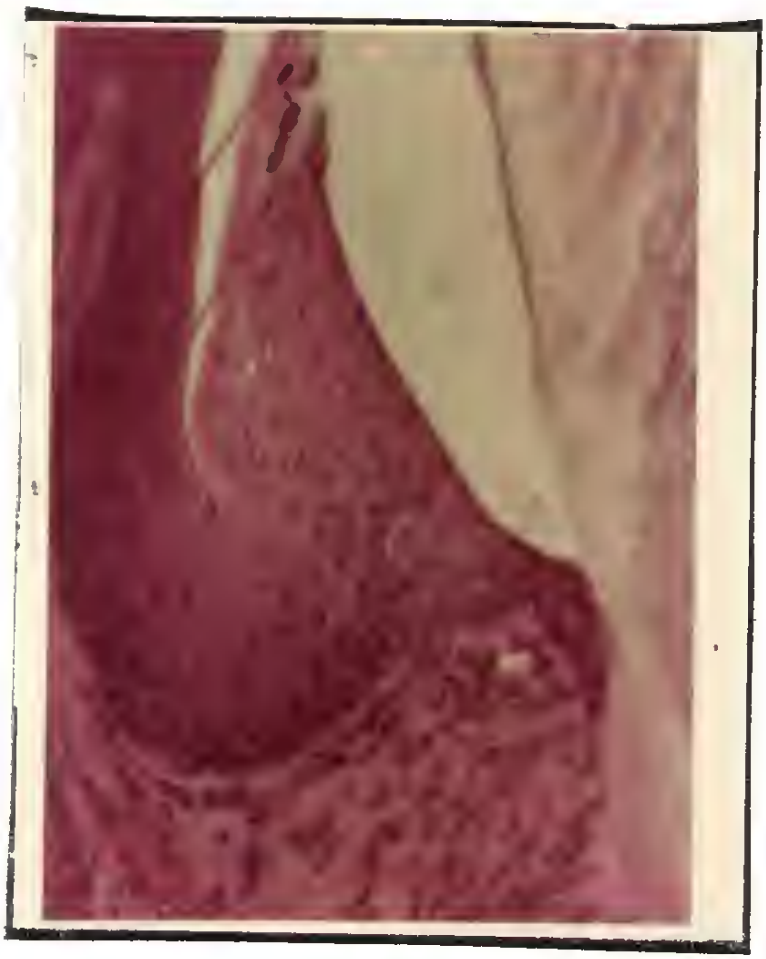

Figure 26. A typical sagital section from infected mice (1.6×10^7 CFU) on a hard diet. These animals are thirteen weeks post-infection.

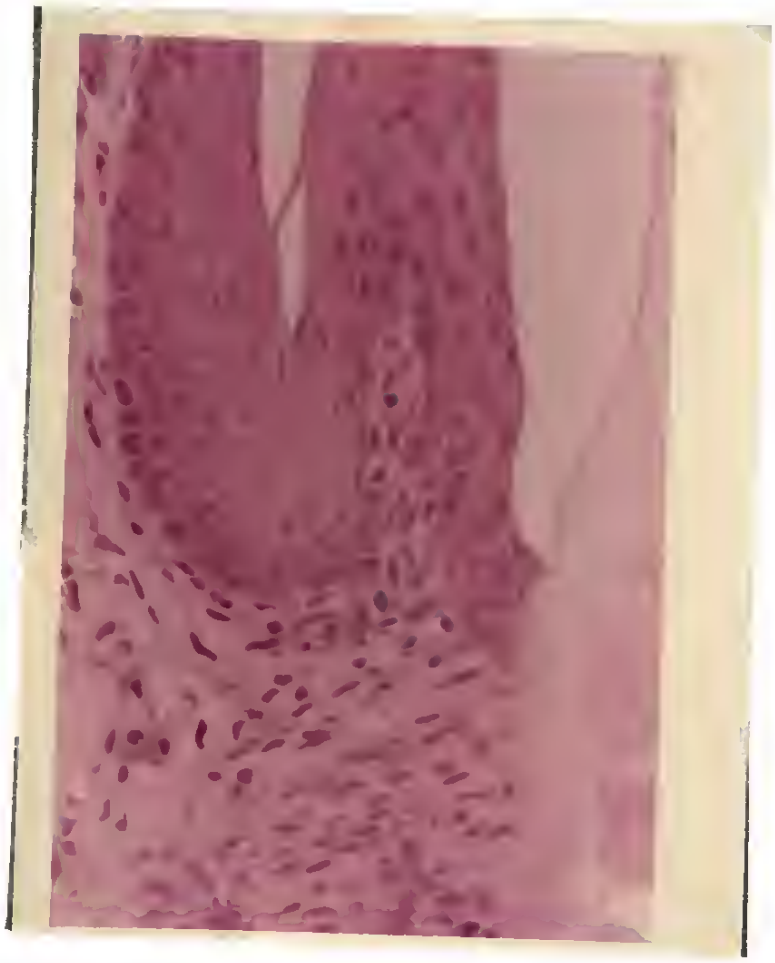

Figure 27. A typical sagital section from uninfected mice on a soft diet. These animals are thirteen weeks post-infection.

Figure 28. A typical sagital section from infected mice (1.6×10^7 CFU) on a soft diet. These animals are thirteen weeks post-infection.

Figure 29. A typical sagital section from infected mice (1.6×10^7 CFU) on a soft diet. This section is from an area apical to previous sections and is at the tip of the crevicular epithelium. These animals are thirteen weeks post-infection.

with cells not seen in the normal tissue. These cells were found throughout the crevicular epithelium as single cells and as large clumps. This aggregation of cells was s especially prevalent along the border of the enamel-soft tissue interface. The epithelial tissue was disrupted by the presence of these inflammatory cells. No inflammatory cells were present in the gingiva.

Analysis of Bacterial Infection in Hyperimmunized Animals

From the above data, an immune response was crucial to the development of bone loss in periodontitis. It would follow, therefore, that an increased rate of bone loss would occur in hyperimmunized animals. To test this hypothesis, 20 mice were immunized with A. viscosus T14V, as described above. These animals and a group of 20 unimmunized animals were inoculated with 1.6×10^8 CFU of A. viscosus T14VJ1 and placed on the soft, high-carbohydrate diet. Animals were sampled, as above, at 2-week intervals. Colonization in the hyperimmunized animal's oral cavity was not detected while the normal, unimmunized animals had the same rate of tooth colonization as was shown in the earlier experiments at 2 and 4 weeks. These data suggest that the preimmunization was preventing tooth colonization and this experimental design was not the proper one to ask this question.

The experiment design of the above work was changed to overcome the colonization problem, so that it could be

asked, if hyperimmunized animals had a higher rate of bone loss. In this protocol, hyperimmunized animals, as discussed earlier, were already colonized with A. viscosus T14VJ1. Forty animals were inoculated with 1.6×10^8 CFU of A. viscosus T14VJ1, placed on the soft diet, and, at 1 month post-inoculation, 20 of them were hyperimmunized with A. viscosus T14V, intraperitoneally. At four weeks of immunization, these two groups of mice were sampled and the hyperimmunized animals were no longer colonized with A. viscosus T14VJ1, while the nonimmunized animals were. These results suggested that hyperimmunization prevents colonization of the bacteria to the tooth surface, and, more importantly, cures the animal.

DISCUSSION

The overall aim of this work has been two-fold. First, the Balb/c mouse was analyzed for its possible usefulness as a model for periodontal disease. Second, the immune response to bacterial antigens was evaluated for its role in inducing the characteristic inflammation and bone loss associated with periodontal disease.

For the application of Balb/c mouse in a model system, for periodontal disease, three requirements had to be met. First the periodontopathogen to be studied, A. viscosus T14V, had to colonize the tooth surface of the infected mice. Second, the Balb/c mouse had to respond immunologically to the bacterial antigens. Third, the Balb/c mouse had to undergo bone loss after infection.

This study established that upon oral inoculation of mice with A. viscosus T14V, the bacteria colonized the teeth of the animals (Table 1). This colonization was profoundly affected by a number of variables. The type of nutrition of the mouse was especially important in establishing an infection. The diet had a nutritional effect on the oral microflora. Food residues in the mouth, or the

surface of the tongue and teeth, within the pits and fissures of molars and in the gingival crevices are used as a substrate that enriches the environment and selects certain microorganisms and possibly inhibits others (88). The initial establishment of a strain of bacteria under dietary stimulus is followed by an increase in their numbers and in their metabolic activity, which tends to depress the implantation and colonization of other organisms. The equilibrium of the oral flora can be controlled and modified to a large degree by the composition of nutrients in the diet (88, 89). There are probably a number of factors which play a role in this effect.

The diet locally affects the oral tissue in the following ways: a) by the texture of the foods, which will influence the force used in mastication and determine the stress placed upon the periodontium, b) by its composition, which will enrich the local environment and to some extent determine the type of organisms that will implant, colonize, and grow at selected sites, c) the texture of the diet influences the residency time of food particles in the mouth and their utilization by the oral flora. The consistency of the diet may be of great importance in enabling the bacteria to remain on the tooth surface. The mechanical action of the hard-chow diet could be removing the bacteria from the tooth surface in a way the soft-chow diet could not.

Such an effect would be consistent with the finding of Krasse and Brill (90), who found that the consistency of the diet had a profound effect on bacteria in the gingiva of beagle dogs. They concluded that the difference was due to less vigorous mastication by dogs on the soft diet.

The high carbohydrate content of the soft-chow diet may be providing added nutrients to the bacteria residing in the mouth (91, 92). The hard-chow mouse diet does not contain the large amount of carbohydrates present in the soft diet and therefore does not enrich the oral environment in the same way as the soft diet.

Colonization was also dramatically affected by the age of the mouse. Mice (before 2 months of age) showed an increasing susceptibility to colonization by A. viscosus T14V, as they age (Figure 2). This effect plateaued with time, eventually decreasing after 4 months of age. Brecher and van Houte have reported that this same effect can be shown in rats infected with A. viscosus (93). Their results indicate that this change is unaffected by diet, antibody, or salivary molecules. They suggest that the difference may be due to the presence of pits and fissures on the tooth. A. viscosus has been shown to heavily colonize such areas (94, 95). This same age related phenomenon has been shown for other bacterial species (96).

The fact that A. viscosus T14VJ1 cells could not be detected anywhere but the tooth surface is particularly

important if the Balb/c mouse is to be an effective model for studying the host response to the resident microflora (Table 2). If the bacteria had colonized other sites, it would not have been possible to solely establish the role of oral colonization in affecting an immune response. This specificity for the oral environment by A. viscosus is in agreement with results of other workers who have described A. viscosus colonization solely on the tooth surface (97, 98).

By immunoelectrophoresis, it was established that the four characteristic antigens of A. viscosus were present in the bacteria from infected mice (Figure 13). Although all four antigens were present, there was a reduction in the amount of antigen present in late samples of the bacteria. Studies indicate that infection of gnotobiotic rats with Streptococcus mutans strains results in rapid selection of antigenically altered mutants (99). The proportion of isolates with altered antigenic composition increased with time following initial inoculation. The rate at which selection of the antigenic variants occurred could be dramatically influenced by prior immunization of the host with the infecting organism. These authors suggested that the immune system provided a major selective factor for antigenic variation in vivo. Such an effect could account for the change in antigens seen in the samples tested.

This study showed that the Balb/c mouse responded immunologically to an i.p. injection of A. viscosus antigens (Table 3, Figure 3). There was a large increase in serum antibody and lymphoblast response to A. viscosus upon i.p. immunization with the bacteria. The route of antigen exposure for these studies was different than that expected from an oral colonization with the bacteria. These results, therefore, demonstrate that the mouse strain was capable of mounting both a humoral and cell mediated response to A. viscosus antigens. These results do not mean that this is the expected response in an orally infected animal.

When the data are closely examined, the i.p. immunized animals have undergone a change in optimal dose of A. viscosus antigens. This may represent an artifact of the system or reflect a real change in the dose-response curve.

Another important component in development of a model for periodontal disease was that the animal manifests pathology characteristic of periodontitis. It was particularly interesting that animals on both hard and soft diets developed a substantial amount of bone loss (Figure 4). It had previously been shown that bacteria colonize at different levels depending on the diet; therefore, the diet must have had a profound effect on the development of periodontal disease. This concept would support work that has shown that the development of periodontal disease in rodents can be profoundly affected by the diet used (100, 101, 102).

They found that mastication of the hard diet produced marked tooth wear. This was also prevalent in the Balb/c mouse described above. The rough physical consistency of the hard diet led to extensive food impaction, inflammation and severe recession of gingival tissue. The animals on the soft diet also had inflamed gingiva but there was no evidence of epithelial penetration nor of drastic tooth wear. This suggested that the periodontal bone loss and gingival inflammation seen in animals on the two types of diets in this study may be due to two slightly different mechanisms. One (the hard diet) induced periodontal stress while the other (the soft diet) caused large bacterial accumulation.

In summary, this study established that the Balb/c mouse strain can be a model for periodontal disease studies. The animals could be orally infected, and this infection was specific for the tooth surface. The animals responded immunologically to the bacterial antigens. The animals lost alveolar bone as a result of infection. The results are in agreement with previous work done in other rodent systems and in man. The present model has the added attraction that all three criteria are in one animal model. The result, so far, suggests that a slightly different mechanism may exist for the bone loss seen in hard and in soft food fed animals.

There was difficulty in assessing the role of bacterial cell numbers in inducing periodontal disease in hard-food-fed animals. The number of bacteria recovered vary greatly

from animal to animal. This effect was independent of the amount of inoculum used initially. This point is especially important when attempting to correlate bacterial colonization with the development of the immune response.

The soft-food-fed animals, on the other hand, attained a much higher level of infection and one that was highly reproducible (Figure 12). The numbers of bacteria recovered from the teeth of high-dose and low-dose inoculated animals was similar throughout the course of the experiment, even though there was a 1000-fold difference in size of inoculum. These data suggest that there exists a finite number of oral receptor sites available and that the numbers of bacteria in the low-dose inoculum was sufficient to fill those sites. The increase in numbers of bacteria recovered was most probably due to bacterial aggregation and not to colonization of additional sites on the tooth surfaces.

The cell mediated immune response of the animal to A. viscosus sonic supernatant antigens or to LPS was dependent upon the initial inoculum given the animal. In no set of experiments did a high-dose inoculated animal develop a cell mediated response as measured by ^{3}H-thymidine uptake (Figures 7 and 14). This was initially surprising considering the profound difference in numbers of bacteria recovered from hard and soft food fed infected animals. The only similarities in bacterial cell numbers between the two experimental regimes was the initial inoculum. Therefore, the initial inoculum must be exerting a regulatory role upon

the immune system. Using very high doses (>10^9), others have observed a lack of cell mediated response to sheep red blood cells administered orally (103). Chase (104) was able to show that an oral administration of a large amount of dinitrochlorobenzene to guinea pigs specifically prevented hypersensitivity responses induced by repeated intracutaneous injections of the contact agent. Suppressor T cells and B cells, antigen-antibody complex and free immunoglobulin have all been found capable of initiating and maintaining this unresponsive state (105, 106, 107, 108). Because the lymphoblast assay used in the present studies utilized human serum, antigen-antibody complexes and free immunoglobulin from the mouse serum can probably be ruled out as suppressor mechanisms, unless they are previously complexed with the responding cells. This leaves the role of suppressor T and B cells in the mechanism of unresponsiveness. Suppressor T cells can be ruled out as a mechanism because, when T cells were depleted by anti-theta ascites fluid and complement treatment of splenocytes, the resulting population did not increase their response to either A. viscosus antigens or to LPS (Table 4). It can be argued that the residual population (3%) of T cells left after anti-theta treatment is capable of suppressing the response. The data do not rigorously exclude this possibility. It is possible that the suppressor population is a B cell.

In the groups of animals that did respond to A. viscosus antigens and to LPS, the response varied with the

diet used (Figures 7 and 14). The animals on a hard diet responded in the sixth and the eighth week, but the response decreased subsequently. This effect is similar to that seen when Eikenella corrodens, another known periodontopathogen, was inoculated into germfree rats. No cell mediated response was detected for five weeks and then the animal responded for four weeks and the response dropped to baseline levels (109). There was no bone loss in Eikenella corrodens infected rats until the immune response decreased to baseline levels. These results suggested that the immune system played a protective role in periodontal disease and once the protection was gone, the disease ran rampant. There was one major assumption in these studies that may not be valid. It was assumed that the splenic response measured in these assays was truly reflective of the inflammatory conditions in the local gingival environment. This has not been tested with this diet.

The response to A. viscosus antigens seen in low-dose inoculated animals on a soft diet started at the same time as that seen in hard-diet animals but was sustained throughout the course of the experiment (Figures 14 and 15). This is an important difference between the two experiment conditions since the theory that the immune system was protective would not be valid here. This response is similar to that seen in humans with periodontal disease, and therefore, more closely resembles the condition in man (34). These results would suggest that induction of the immune system was the

mechanism of periodontal damage in soft food fed animals since bone loss was never observed in unresponsive animals on a soft diet.

The influence of immune responses on gingival inflammation has been studied by using immuno-suppressive treatment and immuno-potentiating drugs. Patients having long-term immuno-suppressive treatment show decreased gingival inflammation and this is correlated with negative lymphoproliferative responses to oral micro-organisms (110).

In contrast, the immuno-potentiating drug, Levamisole, induced a significant enhancement of the *in vitro* lymphoproliferative response. This was correlated with a significant increase in the gingival index of inflammation. This is further evidence for the role of the immune system in periodontal disease (110).

When the draining lymph nodes of the gingival area, the cervical nodes, were used in the lymphoblast assay, a response similar to that of the spleen was seen in mice fed soft food (Figure 18). The cells of the cervical lymph nodes responded at eight weeks post-infection and continued throughout the course of the experiment. A splenic unresponsive animal was also cervical lymph node unresponsive to *A. viscosus* sonic supernatant. The local environment was, in fact, mimicked by the splenic response in soft-food-fed animals, although the initial time of induction needs to be delineated. The experiments so far do not indicate which organ responded first.

The need to use the cervical lymph nodes instead of the gingival tissue for analysis of locally reactive cells was one drawback for use of the mouse system. The gingival tissue in the mouse is too small to make it useful in cell isolation studies.

These results would suggest that the diet of the animal was directly responsible for the type of response seen in that animal. The effect of the diet on bacterial cell numbers and on type of colonization has already been discussed, but the diet consistency plays a major role in the condition of the periodontium. The hard food is able to penetrate the epithelium and cause gingival irritation while the soft food cannot (111). When bacteria are present within the hard food, bacteria are presented to the gingiva in a way that is different from the soft food presentation. Bacteria or large antigens of the bacteria would be able to penetrate the epithelial tissue due to the hard food tissue damage. On a soft diet, only small molecules would be able to gain access to the inner gingival tissue by traversing the epithelium. This difference in antigen presentation to the immune system may be sufficient to produce the differing responses observed with the two diet groups.

When the responding cell population in the spleen is investigated with specific antisera and complement, the only cell type found capable of responding is the B cell (Table 4). At no time was a T cell population found which was capable of responding to A. viscosus sonic supernatant antigens in

the lymphoblast assay. This is especially interesting because antigens of A. viscosus have been shown to contain a B cell mitogen for mouse splenocytes (73, 74). The selective response of B cells in the infected mice may be due to the action of this mitogen. These hypotheses also correlate with the observation that the LPS response in responding animals increased over baseline levels and the increase paralleled that seen to A. viscosus antigens. No comparable increase in response was ever observed with Con A in culture.

The possiblity that dental plaque may exert an adjuvant effect in vivo has been raised on the basis that an increase in DNA synthesis and release of MIF by lymphocytes can be induced in vitro not only by related antigens (49). Although purified cell preparations were not examined, B lymphocyte functions were examined by lipopolysaccharide activation for B lymphocytes (49). There was no detectable increase in serum immunoglobulins. This is consistent with the view that dental plaque can act as a polyclonal B cell activator (112). Although LPS is best known for its adjuvant properties, it may also suppress immune responses if given before the antigen (113). High zone B cell tolerance can be induced in mice by polysaccharides (114). Low zone tolerance is induced by immunization by levan (115).

It is also possible that the mitogen was not responsible for the response seen in infected animals and that the antigen was one that specifically activated B cells. This has been shown to be the case with repeating capsular

polysaccharides (116). A. viscosus contains an amphipathic molecule and a repeating capsular polysaccharide (117, 118). Both are strong candidates for selective activation of B cells.

It follows that since the low-dose infected animals splenocytes responded more strongly to A. viscosus antigens over time of infection and that this response was primarily a B cell response, increasing levels of anti-A. viscosus antibody should be present in the serum of these animals. At no time was an antibody response to A. viscosus antigens detected in the serum (Figure 20) of animals on a soft food diet. This discrepancy may be due to a number of factors. First, the radioimmunoassay used may not be detecting the antibody. The standard for the assay was mouse antisera produced by intraperitoneal injections of whole bacteria into mice. The antibody made to this immunization protocol may be entirely different from that made to an oral infection of A. viscosus in both specificity and affinity. Second, if the A. viscosus B-cell mitogen is activating cells in the spleen, there may be a polyclonal response to the mitogen. This would mean that there was an increase in many different antibodies without any particular specificity for A. viscosus antigens. Third, since there has been no determination of the affinity needed to detect antibody in the RIA, it is possible that the affinity of this antibody

made to A. viscosus antigens was too low to be detected by the assay. Fourth, the oral administration of the bacteria may have produced a tolerant state in antibody production (119, 120). This explanation fits into the unresponsive animals' lack of response and other workers have shown that an unresponsive antibody production does not necessarily correlate with a responsive cell-mediated response (105). So, there could be no antibody production in an animal with a cell-mediated response to a particular antigen.

Antibodies to a variety of plaque bacteria have been observed in human sera by several investigators (121, 122, 123, 124). This is consistent with the antibody data of animals on a hard diet (Figure 9), even though the percent differences were low.

The data on oral colonization of hyperimmunized mice would suggest that development of an antibody response would, in fact, impede the maintenance of the bacteria in the mouth. It is possible that the ability of the bacteria to colonize without producing an antibody response is an important mechanism of survival for the bacteria.

Gnotobiotic rats immunized parenterally with antigens from Actinomyces viscosus responded to dental plaque produced by the micro-organism with a relatively smaller infiltrate compared with control animals (125). A similarly reduced inflammatory response to initial plaque formation was observed in dogs after parenteral immunization with antigens extracted from plaque grown in animals kept on a soft

diet (126). The authors concluded that serum-derived antibodies might have hampered the diffusion of bacterial antigens through the crevicular epithelium or impeded colonization.

Recent animal experiments have indicated that antibodies present intercellularly in the crevicular epithelium resist the penetration of locally applied antigen (horseradish peroxidase) into the gingiva by the formation of immune complexes in the epithelial interstices (127).

In our system, the difference between the two alternatives was clearly evident. These results suggest that if antibodies had been detected in orally infected mice, then the model as described may not have worked. With the above data, it is not possible to delineate between locally produced glandular immunoglobulins and systemically produced antibodies crossing into the oral cavity.

An essential aspect of periodontal disease is that bone loss occurs (Figures 10 and 21). This is a primary characteristic of periodontal disease. The unifying phenomenon in infected animals on either diet was that bone loss never occurred unless the animal responded to the bacteria. An immune response was essential for bone loss. This was an important finding as it demonstrated that bacterial colonization alone was not sufficient for the induction of periodontal disease. The type of tissue destruction may be different between the two diets. The hard diet creates a lot of food and hair impaction and damages the epithelial

tissue. The soft diet does not create either problem (128). The damage to the tissue in hard diet fed infected animals may be heightened by the diet itself whereas the damage in soft-diet-fed animals represents less damage by diet and can primarily be attributed to the bacterial insult.

The experiments using organ cultures of gingival tissues were similar in cultures from infected or uninfected animals. There are a number of possible explanations for these results. First, this may reflect no true difference in tissue between a normal and an infected animal. This would mean that the disease in mice is unlike that seen in humans. The histological studies suggest that this is not the case. Techniques utilized did not allow the identification of individual cell types, but there was, certainly, an inflammatory response in the soft tissue of infected animals. Second, the tissue collected from three animals may not have been enough to get sufficient antibody levels in the culture fluid. This explanation cannot be excluded. Third, the assay system could not detect the immunoglobulin made. The explanation seems doubtful as a positive serum control was included at all times.

The histological sections of thirteen-week-infected mice clearly revealed an inflammatory response with soft tissue damage in the crevicular epithelium. This was not evident in uninfected control mice of the same age. Subsequent extension of these studies must be designed to evaluate the kinetics of this response. An inflammatory response

in the soft tissue should be evident prior to a response in the lymphoid organs of the host if the primary insult was through the gingival tissue.

The soft tissue disease is clearly different in mouse and man. In man, the gingiva is heavily infiltrated with inflammatory cells upon infection (15). In the mouse, the crevicular epithelial tissue was invaded and damaged but no apparent gingival damage could be seen. This difference in tissue involvement may be due to the difference between the soft tissue in man and mouse. The mouse gingival tissue was heavily keratinized providing more protection to the soft tissue than the unkeratinized human gingiva (129).

Keratinized tissue interfers with the transfer of molecules and therefore could possibly interfere with the movement of metabolites and microbial products, which would normally enter the gingiva in humans and elicit an inflammatory response (130).

The crevicular epithelium showed less keratinization and thus less protection than the gingival epithelium. The bacterial colonization adjacent to the gingival tissue in the mouse may be less of an insult than in man, thereby producing less damage due to the extensive keratinization. The soft tissue of the mouse also differed from man in that the crevicular epithelium is separated from the gingival tissue in the mouse. In man they are one tissue (6). In this way, the separated tissue may become inflamed without any gingival response.

The soft tissue inflammatory cells in infected animals were prevalent in the connective tissue underlying the epithelial tissue and adjacent to the alveolar bone. These sections were heavily inflamed and may act as the seeding points from which the cells in the epithelium emigrate. This movement of inflammatory cells from the deeper connective tissues to the epithelium is similar to that seen in man (15).

The inflammatory cells prevalent in the crevicular epithelium clumped together in the thirteen week post-infection samples. This clumping was especially apparent on the border to the gingival crevice where a large accumulation of bacteria colonized. This same effect is observed in gnotobiotic rats monoinfected with Actinomyces naeslundii (129). In these infected animals, inflammatory cells clumped with each other as well as with fibroblasts and macrophages. In the animals, the predominant inflammatory cell was always the polymorphonuclear leukocyte with a lesser accumulation of plasma cells and lymphocytes.

Circulating IgA blasts home preferentially to glandular sites such as the gut, independently of antigen attraction (131). Glandular homing apparently takes place also for other blast classes, since even most intestinal IgG-producing cells are J-chain positive. In contrast, less than 1% of the gingival IgG cells contain J chain. These gland-associated and gingival immunocyte populations are basically different and are probably established by different

mechanisms. While the former is maintained by a continuous supply of circulating cells from an early clonal differentiation phase, the gingival lesion rather appears to contain cells from established clones (131).

Mononuclear cells obtained from crevicular washings show a great preponderance of B lymphocytes and contain some blast cells (132). The seeding of lymphoid cells into an inflammatory focus has been assumed to be a random process (133, 134, 135) but experiments suggest that antigen can specifically select a small sub-fraction of cells from the recirculating pool (135, 136). In addition, however, affinity for inflamed tissue is shown by cells newly stimulated in immune responses (blasts), regardless of specificity (137, 138, 139). When such cells mature to plasma cells in the gingiva, one may expect a considerable production of non-reactive immunoglobulins, that is, antibody moledules not directed against dental plaque antigens.

If circulating blasts home to both inflammatory sites and glandular regions, regardless of antigen specificity, then there must be a selection depending on their tissue origin. It has been shown that T blasts from peripheral lymph nodes move readily to inflamed skin (140). Similar observations have been made for IgA producing B cells (141). It has been difficult to determine whether selectivity is involved in the emigration or the local retention of circulating blasts. Two possibilities can explain the characteristics of the gingival Ig-producing immunocytes: they are

either derived as blasts from secondary immune responses in regional lymph nodes and mature locally or they belong to established clones that continue to proliferate in the gingiva because of persistent stimulation by antigens and mitogens from the plaque. Both aspects may be relevant, perhaps depending on the stage of the gingival lesion.

A possible mechanism of action in the mouse A. viscosus model for periodontal disease follows from the data presented here. The initial inoculum colonizes the tooth surface and also provides an immunoregulatory signal via the mesenteric lymph nodes and Peyer's patches. These immunoregulators migrate to the peripheral lymphoid organs such as the spleen and peripheral lymph nodes. The data suggest that these are B cells. At 6-8 weeks post-infection, the traumatizational colonization of the bacteria on oral epithelium is sufficient to stimulate a focus of inflammation in the oral tissue. This insult on the epithelium would vary according to the diet used. On a hard diet, the tissue is severely traumatized by the food allowing passage of large molecules across the epithelium. On a soft diet, stimulation has to be via a low-molecular weight molecule migrating across the epithelium. In either case, this provides the initial immunostimulatory signal to the host and, in low-dose infected animals, a response is detected. The type of response has already been programmed by the seeding of the immunoregulatory cells to the peripheral lymphoid organs (e.g. cervical lymph nodes). In responsive animals,

the gingival tissue becomes inflamed with cells which respond to the antigen. In animals on a hard diet, the unregulated assault overwhelms the lymphoid cells and produces a tolerant state to the antigens after 8 weeks. In animals on a soft diet, the assault is regulated by the intact epithelium thereby providing a continuous antigenic exposure to the soft tissues until the epithelium is damaged. The responsive cells migrate to the cervical lymph nodes and to the spleen. Since there is a constant traffic flow of cells between lymphoid organs, each organ now becomes a reservoir of responsive cells and seed each other.

In unresponsive animals, there are two possibilities upon insult of the periodontium. An inflammatory infiltrate could populate the gingival tissue but not respond to the antigens of A. viscosus. Second, there is no inflammation due to the earlier immunoregulatory signal. The data do not support either hypothesis.

The inflammation in responsive animals is responsible for the damage to the tissue since unresponsive animals do not develop bone loss. This damage can be through lymphokine production or antigen-antibody complexes that destroy the tissue directly or via other inflammatory cells (i.e. polymorphonuclear leukocytes or macrophages).

Such a model is based on a number of assumptions. First, that an immune response to a complex set of antigens can be regulated by the oral dose. This work has been done by others using red blood cells as immunogens (103).

Second, that immunoregulatory cells can be produced in the mesenteric lymph nodes and Peyer's patches. Such immunoregulatory cells have been found in these organs (142, 143). Third, that these immunoregulatory cells, specifically B cells, can migrate from the mesenteric lymph nodes and Peyer's patches to the spleen and peripheral lymph nodes, especially the cervical lymph nodes. There is no precedent for such a finding but the Balb/c mouse provides an adequate animal for analyzing the traffic patterns of these cells. Fourth, that the diet alters the amount of antigen exposed to the epithelial tissue. This has not been established but this model provides an opportunity to analyze the question of antigenic presentation. Fifth, that the local inflammation seen in responsive animals is different in unresponsive animals. This question is presently being studied with histological sections of both tissues. Sixth, that responsive cells produce lymphokines and/or antibody and the unresponsive cells do not. This is easily testable within our system with in vitro tissue culture techniques.

As in any experimentation, development of this mouse - A. viscosus model for periodontitis has led to a few answers and many more questions. The nature of the immunodominant antigen(s) (mitogen) in the bacteria is still unknown but is important in understanding the mechanism of response of the host. The mechanism of unresponsiveness in high-dose inoculated mice is clearer but the cell type responsible still has to be characterized. The paradox of activated B

cells and an undetectable serum antibody still needs to be investigated. The system can also be used to analyze the role of specific cell types in the development of the disease process.

Finally, the development of the mouse - A. viscosus model provides an interesting opportunity to study not only a localized oral inflammatory response to a chronic bacterial infection, but it is a possible model for analysis of cellular communication and cooperation between the local mucosal tissue and the generalized immune system.

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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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